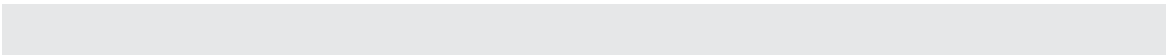


Handbook of Nanotoxicology, Nanomedicine and Stem Cell Use in Toxicology



Handbook of Nanotoxicology, Nanomedicine and Stem Cell Use in Toxicology

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Preface

The preface of our book, *Nanotoxicity: From In Vivo and In Vitro Models to Health Risks*, published in 2009, said in its ending paragraph that “Nanotechnology is an emerging new multidisciplinary field of science, and, therefore, there is a risk of change in its rapid development in the near future.” The rapidly developing nature of this scientific discipline carries the risk of being out of date in certain areas of research and development. Since the publication of our book in 2009, we have witnessed exponential growth of this emerging new nanotechnology. Nanomaterials have become the products of a modern industrial revolution that develops extremely small-sized, lightweight and high-strength materials for a variety of purposes. These nanomaterials are used in a wide range of consumer products such as foods, cosmetics, pharmaceuticals, medicines, and medical devices leading to extensive daily human exposure. Therefore, their unknown effects on human health have become a great public concern, which has given birth to new scientific disciplines such as “Nanotoxicology” and “Nanomedicine.” These new scientific disciplines investigate the issues related to toxicity and environmental impact of nanomaterials on human health.

The stem cells are the origin of all cells in multicellular organisms. They can divide and differentiate into any specific cells. In recent years, these ubiquitous cells have become a very important research tool in biology, medicine, and toxicology. Therefore, the study of stem cells is an emerging new scientific discipline and their use in toxicology and medicine is a rapidly developing new area of research.

The importance of recent research focus on nanotoxicology, nanomedicine, and stem cells is evidenced by the increasing number of contributions

published each year. It becomes increasingly clear that developments in these areas of research are moving so rapidly that new means are needed to report the status of current ongoing up-to-date research activities. Our new book is an attempt to provide such a vehicle for up-to-date information. This book represents a collaborative effort by leading internationally recognized scientists to compile together their own contributions to the latest developments in these emerging areas of research in current modern scientific fields.

The main purpose of this book is to assemble up-to-date, state-of-the-art information on nanotoxicology, nanomedicine, and stem cells presented by internationally recognized experts in these emerging new areas of research in a single edition. This book will provide an insight into the current trends and future directions of research in these rapidly developing scientific fields. Therefore, we sincerely hope that this book will provide a comprehensive and authoritative source of current up-to-date information on these areas of research and prove useful to the scientists interested in these scientific disciplines throughout the world for years to come. It is our sincere hope that the information presented in this book will serve as a stimulus to all the investigators interested in these areas of research. In addition, it should be of interest to a variety of other scientific disciplines including toxicology, medicine, and pharmacology, as well as food, drug, and other regulatory sciences.

Saura C. Sahu
Daniel A. Casciano
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I lovingly dedicated this book to:

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writing, and editing this book.

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Abbreviations and Acronyms

AA	ascorbic acid	BM-MSC	bone marrow mesenchymal stem cell
ACDC	adherent cell differentiation and cytotoxicity	BMI	body mass index
ADMA	asymmetric dimethylarginine	BMP	bone morphogenetic protein
ADMET	absorption, distribution, metabolism, excretion, and toxicity	BRB	blood–retina barrier
ADP	adenosine diphosphate	BRL	buffalo rat liver
AgNPs	silver nanoparticles	BSA	bovine serum albumin
AgAc	silver acetate	Caco-2	colon carcinoma
AgNO	silver nitrate	cAMP	cyclic adenosine monophosphate
AJ	adherens junction	CBM	carbon-based material
ALL	acute lymphocytic leukemia	CBMN	cytokinesis-block micronucleus assay
AML	acute myelogenous leukemia	CD	Crohn's disease
AML	acute myeloid leukemia	CD	cyclodextrin
ANOVA	analysis of variance	CCD	charge-coupled device
anti-VEGF	anti-vascular endothelial growth factor	cell-SELEX	cell-based systemic evolution of ligands by exponential enrichment
AP	action potentials	CF	cystic fibrosis
APBS	adaptive Poisson–Boltzmann solver	CFDA	carboxyfluorescein diacetate
APOE	apolipoprotein E	cGMP	cyclic guanosine monophosphate
ARPE	arising retinal pigment epithelium	CHO	Chinese hamster ovary
ARVD/C	arrhythmogenic right ventricular dysplasia/cardiomyopathy	CK7	cytokeratine 7
ASC	adult stem cell	CLDN	claudin
ASGP-R	asialoglycoprotein receptor	CLIO	cross-linked iron oxide
ATP	adenosine triphosphate	CM	cardiomyocytes
AuNP	gold nanoparticle	CMLC	cardiomyocytes-like cell
BAL	bronchiolar lavage	CMV	cytomegalovirus
BAP	benzo-[a]-pyrene	CNS	central nervous system
BBB	blood–brain barrier	CNT	carbon nanotube
BCNU	1,3-bis(2-chloroethyl)-1-nitrosourea	COPD	chronic obstructive pulmonary disease
BCSFB	blood–cerebrospinal fluid barrier	CpG	cytosine–phosphate–guanine
bFGF	basic fibroblast growth factor	CPVT	catecholaminergic polymorphic ventricular tachycardia
		CS	citrate

CSC	cancer stem cell	ESC	embryonic stem cell
CTAB	cetyltrimethylammonium bromide	EST	embryonic stem cell test
CYP	cytochrome P450	EURL ECVAM	European Union Reference Laboratory for Alternatives to Animal Testing
2D	two-dimensional		
3D	three-dimensional	f-PEG-DSPE	folate-conjugated PEGylated distearoylphosphatidylethanolamine
DCF	dichlorofluorescein		
DHFR	dihydrofolate reductase		
DIC	differential interference contrast	FACS	fluorescent activated cell sorting
DIVEMA	divinyl ether and maleic anhydride	FACS-EST	fluorescence-activated cell sorting embryonic stem cell test
DLPC	dilauroylphosphatidylcholine	FBS	fetal bovine serum
DLS	dynamic light scattering	FCS	fetal calf serum
DMEM	Dulbecco's Modified Eagle Medium	FDA	Food and Drug Administration
DMPC	dimyristoylphosphatidylcholine	FeBAD	fetal basis of adult disease
DMSO	dimethyl sulfoxide	FETAX	frog embryonic teratogenicity assay— <i>Xenopus</i>
DNMT	deoxyribonucleic acid methyltransferase	FGF	fibroblast growth factor
DOX	doxorubicin	FITC	fluorescein isothiocyanate
DSB	double-stranded break	FRET	fluorescent resonance energy transfer
DUB	deubiquitinating enzyme		
EB	embryoid body	GAA	acid alpha-glucosidase
EBPL2	elastin-binding protein ligand 2	GABA	γ -aminobutyric acid
EBs	embryoid bodies	GC/MS	gas chromatography–mass spectrometry
EC	Embryonic carcinoma	GCV	ganciclovir
ECM	extracellular matrix	GECI	genetically encoded calcium indicator
ECVAM	European Centre for the Validation of Alternative Methods	GF	growth factor
EF1 α	elongation factor-1 alpha	GFP	green fluorescent protein
EG	embryonic germ line	GI	gastrointestinal
EGCG	epigallocatechin-3-gallate	GIMIC	gauge including magnetically induced current
EGF	epidermal growth factor		
EGFR	epidermal growth factor receptor	GIT	gastrointestinal tract
EHT	engineered heart tissue	GNP	gold nanoparticle
ELISA	enzyme-linked immunosorbent assay	GO	gene ontology
EMA	European Medicines Agency	GP	glycerophosphate
EMT	epithelial-to-mesenchymal	GP-Av-bEGF	gelatin nanoparticles conjugated to avidin and functionalized with biotinylated epidermal growth factor
EPA	Environmental Protection Agency		
EPR	electroparamagnetic resonance	GSH	glutathione
EPR	enhanced permeability and retention	GT-SPE	glycerol triacrylate-spermine
ES	embryonic stem	GTX	genotoxic carcinogens
		GUI	graphical user interface
		H3K4	histone 3 lysine 4

hAMC	human amniotic mesenchymal cell	INAA	instrumental neutron activation analysis
HAT	histone acetyltransferase	IONP	iron oxide nanoparticle
HCC	hepatocellular carcinoma	iPS	induced pluripotent stem
HDAC	histone deacetylase	iPSC	induced pluripotent stem cell
HEK	human embryonic kidney	ISDD	<i>in vitro</i> sedimentation, diffusion, and dosimetry
Hep	human embryonic progenitor	ISO	International Organization for Standardization
HER2	human epidermal growth factor receptor 2	iv	intravenous
hES	human embryonic stem	IVM	<i>in vitro</i> methods
hESC	human embryonic stem cell	JaCVAM	Japanese Center for the Validation of Alternative Methods
HGPS	Hutchinson-Gilford progeria syndrome	JAK-STAT	Janus kinase-signal transducer and activator of transcription
hiPSC	human induced pluripotent stem cell	JNK	c-Jun N-terminal kinase
HIV	human immunodeficiency virus	KDR	kinase insert domain receptor
HKMT	histone lysine methyltransferase	67LR	67-kDa laminin receptor
HMT	histone methyltransferase	KGF	keratinocyte growth factor
HP	heterochromatin protein	KoCVAM	Korean Center for the Validation of Alternative Methods
HP1	heterochromatin protein 1	LAL	limulus amebocyte lysate
HPMA	<i>N</i> -(2-hydroxypropyl) methacrylamide	LC-MS	liquid chromatography–mass spectrometry
HSF	heat shock factor	LDH	lactose dehydrogenase
HUCB-NSC	human neural stem cell line derived from umbilical cord blood	LDVC	live/dead viability/cytotoxicity
HUVEC	human umbilical vein endothelial cell	LHRH	luteinizing hormone-releasing hormone
i.p.	intraperitoneal	LIF	leukemia inhibitory factor
i.v.	intravenous	LPS	lipopolysaccharide
i/oBRB	inner and the outer blood–retinal barrier	LQT	long QT
ICATAM	Canadian International Co-operation on Alternative Test Methods	LQTS2	long-QT syndrome 2
ICATM	International Co-operation on Alternative Test Methods	LUC	luciferase
iCM	induced cardiomyocyte	MA	multicellular aggregate
ICM	inner cell mass	MALDI-TOF	matrix-assisted laser desorption/ionization–time of flight
ICP-MS	inductively coupled plasma mass spectrometry	MBM	metal-based material
IGERT	Integrative Graduate Education and Research Traineeship	MDR	multidrug resistance
IHCP	Institute for Health and Consumer Protection	MDR1	multidrug resistance protein 1
IL	interleukin	MEF	mouse embryonic feeder
IL-3	interleukin-3	MEF	mouse embryonic fibroblast
im	intramuscular	mES	mouse embryonic stem
		mESC	mouse embryonic stem cell
		mESC	murine embryonic stem cell
		mEST	mouse embryonic stem cell test

α MHC	alpha myosin heavy chain	NIDDK	National Institute of Diabetes and Digestive and Kidney
MHC	myosin heavy chain		
MION	monocrystalline iron oxide nanoparticle	NIH	National Institutes of Health
		NIR	near infrared
MLN	mesenteric lymph node	NIST	National Institute of Standards and Technology
MN	micronuclei		
MPPD	multiple-path particle dosimetry	NK	natural killer
		NM	nanomaterial
MPS	mononuclear phagocytic system	NMDA	<i>N</i> -methyl-D-aspartate
		NMR	nuclear magnetic resonance
MPTP	mitochondria permeability transition pore	NOEL	no observable effect limit
		NP	nanoparticle
mRFP	monomeric red fluorescent protein	NR	neutral red
		Nrf2	nuclear factor erythroid 2-related factor 2
MRI	magnetic resonance imaging		
MRP	multidrug resistance-associated protein	NRK	normal rat kidney
		NuRD	nucleosome remodeling and deacetylase
MS	mainstream		
MS	mass spectrometry	OAT	organic anion transporting
MSC	mesenchymal stem cell	OECD	Economic Co-operation and Development
MSN	mesoporous silica nanoparticle	OECD	Organization for Economic Co-operation and Development
MTD	maximum tolerated dose		
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)- <i>2H</i> -tetrazolium	P(Asp)	poly(aspartic acid)
		PAE	poly(β -amino ester)
		PAMAM	polyamidoamine
		PB	<i>piggyBac</i>
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenoltetrazolium bromide	PBCA-NP	polybutylcyanoacrylate nanoparticle
		PBMC	peripheral blood mononuclear cell
MUA	mercaptoundecanoic acid		
MWCNT	multiwalled carbon nanotube	PCA	principal component analysis
NC	noncarcinogens	PCP	personal care product
NCL	Nanotechnology Characterization Laboratory	PCR	polymerase chain reaction
		PDGFRA	platelet-derived growth factor receptor- α
NDBSU-hESC	nondynamic blebbing single unattached human embryonic stem cell	PDLLA	poly(D,L lactide)
		PDSC	placental-derived stem cell
NGTX	nongenotoxic carcinogens	PE	puff equivalent
NHDF	normal human dermal fibroblast	PEG	polyethylene glycol
		PEG-USPIO	polyethylene glycol-ultrasmall
NODE	Nanog and Oct4-associated deacetylase		superparamagnetic iron oxide
NICEATM	National Toxicology Program	PEI	polyethylenimine
ICCVAM	Interagency Center for the Evaluation of Alternative Toxicological Methods and Interagency Coordinating Committee on the Validation of Alternative Methods	PEO	poly(ethylene oxide)
		PGD	preimplantation genetic diagnosis
		PGK	phosphoglycerate-kinase
		PGK-HSVtk/GCV	ganciclovir
		PI	propidium iodide

piRNA	piwi-interacting ribonucleic acid	siRNA	small interfering ribonucleic acid
PLA	polylactic acid	SIRPA	signal regulatory protein alpha
PLGA	polylactic- <i>co</i> -glycolic acid	SMC	smooth muscle cell
PLL	poly-L-lysine	SOD	superoxide dismutase
POU	Pit-Oct-Unc	SP ICP-MS	single-particle inductively coupled plasma mass spectrometry
PP	Peyer's patches		superparamagnetic iron oxide nanoparticle
PPBA	poly(4-phenyl-1-butanoate) l-aspartamide	SPION	sidestream
PPI	polypropylenimine	SS	stage-specific embryonic antigen-1
PRC2	polycomb repressive complex 2	SSEA-1	stable enterotoxin
PRMT	protein arginine methyltransferase	ST	supraaavalvular aortic stenosis
PS	polystyrene	SVAS	single-walled carbon nanotube
PSMA	prostate-specific membrane antigen	SWCNT	transcription activator-like effector nuclease
PT/PA	photothermal/photoacoustic	TALEN	transactivator of transcription
PTM	posttranslational modification	TAT	Torsade de pointes
PTX	paclitaxel	TdP	transmission electron microscopy
PVP	polyvinylpyrrolidone	TEM	transcription factor
QD	quantum dot	TF	transferrin
qRT-PCR	quantitative real-time polymerase chain reaction	Tf	transforming growth factor
QSAR	quantitative structure–activity relationship	TGF	transforming growth factor-beta
RAPD	random amplified polymorphic deoxyribonucleic acid	TGF β	tetrahydrofuran
RBC	red blood cell	THF	tight junction
RBL	rat basophilic leukemia	TJ	titanium dioxide nanoparticle
RES	reticuloendothelial system	TNP	tumor necrosis factor- α
RGC	retinal ganglion cell	TNF- α	total parenteral nutrition
RGD	arginine-glycine-aspartic acid	TPN	mono-sulfonated triphenylphosphine
RNS	reactive nitrogen species	TPPMS	Timothy syndrome
ROA	route of administration	TS	terminal deoxynucleotidyl transferase dUTP nick end labeling
ROS	reactive oxygen species	TUNEL	ulcerative colitis
RT-PCR	reverse transcription polymerase chain reaction	UC	United States Food and Drug Administration
SAHA	suberoylanilide hydroxamic acid	USFDA	ultra small paramagnetic iron oxide
SAM	S-adenosyl methionine	USPIO	video bioinformatics
SB	Sleeping Beauty	VBI	vascular cell adhesion molecule 1
SCID	severe combined immunodeficiency	VCAM-1	vascular endothelial growth factor receptor 2
SCLC	small cell lung cancer	VEGFR-2	Williams–Beuren syndrome
SCNT	somatic cell nuclear transfer	WBS	
SDS	sodium dodecyl sulfate		
SET	su(var), enhancer of zeste, trithorax		

WST-1	4-[3-(4-iodophenyl)- 2-(4-nitrophenyl)-2 <i>H</i> - 5-tetrazolio]-1,3-benzene disulfonate	ZEBET	National Centre for Documentation and Evaluation of Alternative Methods to Animal Experiments
XTT	2,3-bis-(2-methoxy-4-nitro- 5-sulfophenyl)-2 <i>H</i> - tetrazolium-5-carboxanilide	ZFN	zinc finger nuclease
YFP	yellow fluorescent protein	ZnO-NP	zinc oxide nanoparticle

Part One

Nanotoxicology

Part One

Nanotoxicology

Testing Nanotoxicity: An Update of New and Traditional Methods

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1 INTRODUCTION

The exploitation of engineered nanomaterials (NMs) in many technological fields is partly rapidly evolving and partly well consolidated. Nanotoxicity, the dark side of the nanotechnology revolution, became a matter of concern since 1990. More than a decade later, three nonconnected and acute occurrences of toxic reactions in humans, possibly related to exposure to NMs, were reported (Kanarek, 2007; Seaton, 2006; Song, Li, and Du, 2009; Toyama *et al.*, 2008). In all these cases, however, the cause–effect relationship was suggested, but not proved. While sporadic, these adverse effects were severe enough to enhance the alert about the safety of NMs. Currently, nanotoxicology had already evolved into an independent discipline and included aspects of preventive and occupational medicine.

Khatri *et al.* (2013) recently exposed normal volunteers to nanoparticles (NPs) emitted by photocopiers, in conditions mimicking professional exposure, and observed a significant increase of inflammation markers in the upper airways. Sharma *et al.* (2012) associated inhalation of metallic NPs

with whole-body hyperthermia, which causes leakage of the blood–brain barrier (BBB), cerebral edema, and neuronal damage. Whole-body hyperthermia and inhalation of NPs can happen together in professionals who operate in hot, desert areas and are exposed, by inhalation, to ultrafine metallic and silica particles produced by diesel exhaust or gunpowder explosion (Sharma *et al.*, 2012). NPs of metals, lanthanides, and uranium are common by-products of industrial or war activities; devastating consequences can be expected, but not yet demonstrated in human (Petitot *et al.*, 2013). As a result of these findings, nanotoxicology has become a discipline of interest for military medicine as well.

While the terms “nanotoxicology” and “nanotoxicity” firstly appeared in peer-reviewed publication in the years 2004 and 2005 (Service, 2004; Braydich-Stolle *et al.*, 2005), the experimental assessment of the toxicity of new NMs was the subject of papers appeared, although sporadically, as early as in the 1980s. The very first studies concerned the safety of organic polymeric NPs for the release of anticancer drugs (Couvreur *et al.*, 1982; Kante *et al.*, 1982) and that of iron

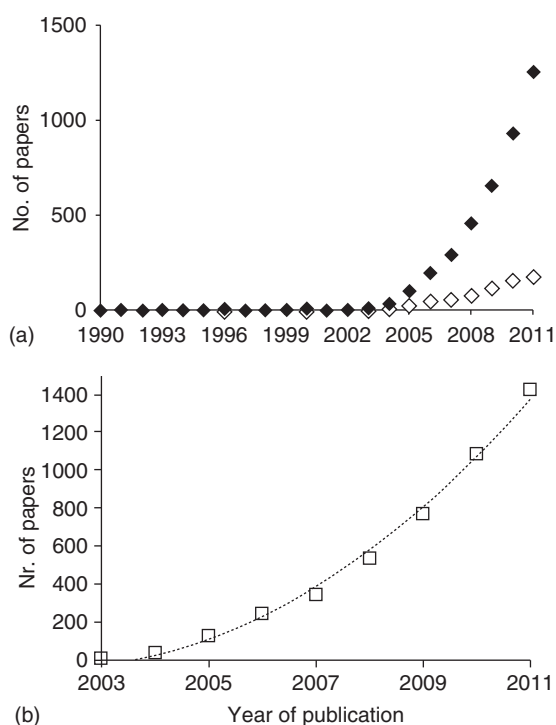


Figure 1. (a) The increasing number of toxicity or toxicological studies of nanomaterials, deposited at the NCBI PubMed during the period 1990–2011. Papers with experimental content (◆) and those with a more general content (◇) are separately plotted. These include reviews, editorials, opinions, symposia announcements, and similar. (b) The growth of editorial production since the year 2003. Retracted, corrected, and republished or duplicate publications were absent in the database under the considered period. Only one published erratum was found applying these search terms. **Boolean search string:** (toxicity) OR (toxicology) AND nanomaterials.

oxide NPs, whose superparamagnetic properties were exploited for magnetic resonance imaging (Josephson *et al.*, 1990). Figure 1 plots the papers published, in the field of nanotoxicology, during the period 1990–2011 and clearly shows the sporadic nature of production before 2003. Original and experimental works, in particular *in vitro* studies, largely exceeded papers with more general content, such as reviews, opinions, editorials, and others. While still scarce (Figure 2), studies *in silico*, inclusive of computational studies, earned increasing interest and will be therefore discussed aside. Figure 3 illustrates the distribution of published studies according to the chemical nature of NMs.

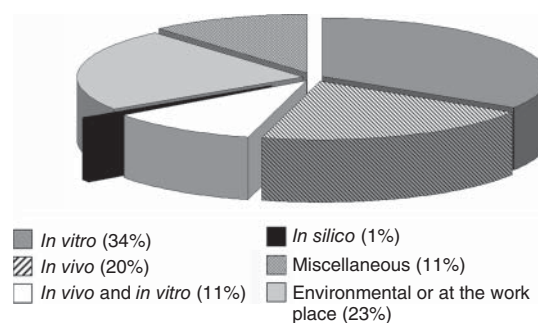


Figure 2. Distribution of published papers describing the toxicity of nanomaterials according to the method of study: *in vitro*, *in vivo* (or combined) experimental studies, environmental together with exposure at the workplace, and *in silico* including computational studies. The miscellaneous category includes reviews, editorials, clinical observations, case reports, and others not falling into the previous groups.

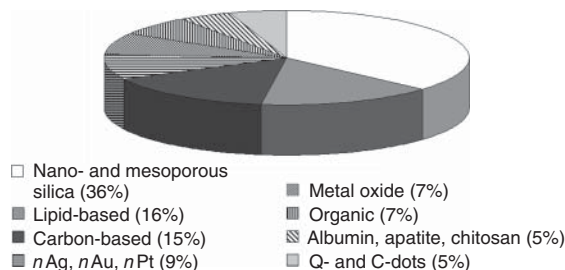


Figure 3. Distribution of published papers describing the toxicity of different types of nanomaterials. Liposomes were not included in the search.

2 THE POINT OF VIEW OF THE REGULATORY AGENCIES

Nanotoxicology deals with the adverse effects possibly occurring as a consequence of the exploitation of nanotechnology, either for the daily life, or after accidental, professional, unavoidable, or incautious exposure to NPs. These cases eventually fail in the fields of ecotoxicology or forensic medicine (Batley, Kirby, and McLaughlin, 2013; Kuempel, Geraci, and Schulte, 2012; Landsiedel *et al.*, 2012; Ling *et al.*, 2012; Lowry *et al.*, 2012). Therefore, the regulatory agencies for the control of environment and health are directly involved in defining NMs and assessing their safety. Tables 1–4 list the documents most recently released and the web references to access them. We recall here, for their importance, the strategies to test the genotoxicity of NMs issued

Table 1. List of documents recently released by the organization for OECD, concerning the regulatory purposes for nanoscience and nanosafety.

Year	Document identification	Content
2010	ENV/JM/MONO(2010)46	List of manufactured nanomaterials and list of endpoints for phase 1 of the sponsorship program for the testing of manufactured nanomaterials: revision
2011	ENV/JM/MONO(2011)12	Current developments/activities on the safety of manufactured nanomaterials
	ENV/JM/MONO(2011)53	Information gathering schemes on nanomaterials: lessons learned and reported information
2012	ENV/JM/MONO(2011)54	National activities on life cycle assessment of nanomaterials
	ENV/JM/MONO(2012)8	Important issues on risk assessment of manufactured nanomaterials
	ENV/JM/MONO(2012)12	OECD Environment, Health and Safety Publications' <i>Series on the Safety of Manufactured Nanomaterials</i>
	ENV/JM/MONO(2011)13	Current developments on the safety of manufactured NMs
	ENV/JM/MONO(2011)14	Inhalation toxicity testing: expert meeting on potential revision to OECD test guidelines and guidance document
	ENV/JM/MONO(2012)40	Guidance for sample preparation and dosimetry for the safety testing of manufactured nanomaterials
2013	DSTI/STP/NANO(2013)3/FINAL	Nanotechnology for green innovation
	DSTI/STP/NANO(2012)22/FINAL	Regulatory framework for nanotechnology in foods and medical products
	NEA/RWM/CLAYCLUB(2013)1	Clay characterization from nanoscopic to microscopic resolution

Table 2. Documents and protocols approved by the ISO.

Year	Identification	Content
2002	ISO 14577-1	Instrumented indentation test for hardness and materials parameters – Part 1: Test method for metallic materials
2007	ISO 14577-4	Instrumented indentation test for hardness and materials parameters – Part 4: Test method for metallic and nonmetallic coatings
	ISO/TR 27628	Ultrafine, nanoparticle, and nanostructured aerosols – inhalation exposure characterization and assessment
2008	ISO/TS 27687	Terminology and definitions for nanoobjects – nanoparticle, nanofiber, and nanoplate
2010	ISO 9277	Determination of the specific surface area of solids by gas adsorption – Brunauer, Emmett, and Teller method
	ISO 10808	Inhalation toxicity testing: characterization of nanoparticles in inhalation exposure chambers
	ISO 29701	Safety testing of NMs intended for cell-based <i>in vitro</i> biological test systems: adaptation and application of the LAL test reagents
	ISO/TR 11360	Methodology for the classification and categorization of nanomaterials
2011	ISO/TS 80004-3	Vocabulary – Part 3: Carbon nanoobjects
	ISO/TS 12805	Materials specifications – guidance on specifying nanoobjects
	ISO/TS 80004-5	Vocabulary – Part 5: Nano/biointerface
	ISO/TR 14187	Surface chemical analysis – characterization of nanostructured materials
	ISO/TR 13121	Evaluation of risk hazard of developing and using manufactured NMs: guidelines to identify, evaluate, address, and communicate the potential risks. Public health, forensic medicine
2012	ISO/TS 12025	Quantification of nanoobject released from powders by generation of aerosols
	ISO/TS 12901-1	Occupational risk management applied to engineered nanomaterials – Part 1: Principles and approaches
	ISO/TR 11811	Guidance on methods for nano- and microtribology measurements
2013	ISO/TS 16195	Guidance for developing representative test materials consisting of nanoobjects in dry powder form

Table 3. List of documents recently released by the US agencies for standardization, concerning the regulatory purposes for nanoscience and nanosafety.

Agency	Year	Document identification	Content
US FDA	2007	FDA Report, Nanotechnology	State of the science of nanotechnology relevant to FDA, safety assessment of NMs
	2008	FDA Nanotechnology Public Meeting	Nanotechnology and dietary supplements
			CYT-6091 (Aurimune™): a model cancer nanomedicine Safety of NMs in food packaging The importance of defining chemical and physical parameters for toxicological testing
	2012	FDA Draft Guidance UCM300927, 2012	Guidance for industry. Assessing the effects of significant manufacturing process changes, including emerging technologies, on the safety and regulatory status of food ingredients and food contact substances, including food ingredients that are color additives
		FDA Draft Guidance UCM300932, 2012	Guidance for industry safety of nanomaterials in cosmetic products
US EPA	2010	EPA/600/R-10/117	Characterizing concentrations and size distributions of metal-containing NPs in waste water
		EPA/600/R-10/129	State-of-the-science. Report on predictive models and modeling approaches for characterizing and evaluating exposure to nanomaterials
		EPA/600/R-10/084 Varner <i>et al.</i> , 2010	Everything <i>n</i> Ag and more Sensors as tools for quantitation, nanotoxicity, and nanomonitoring assessment of engineered NMs
		EPA/600/R-10/105	A feasibility study on the geophysical response to NPs in the subsurface
		EPA/600/R-09/057F	Nanomaterial case studies: Nanoscale titanium dioxide in water treatment and in topical sunscreen (final)
	2011	EPA/600/R-11/096	Screening methods for metal-containing NPs in water
		EPA/600/R-11/097	Laser detection of NPs in the environment
		EPA/600/R-11/107	Guidance to facilitate decisions for sustainable nanotechnology
	2012	EPA/600/R-10/081F	Nanomaterial case study: Nanoscale silver in disinfectant spray
		EPA/600/R-12/043A	Nanomaterial case study: A comparison of multiwalled carbon nanotube and decabromodiphenyl ether flame-retardant coatings applied to upholstery textiles (external review draft)

by the organization recommended by the Economic Co-operation and Development (OECD) (Doak *et al.*, 2012) and the documents of the International Organization for Standardization (ISO), which define the procedure for testing respiratory toxicity of NMs and toxicity for cells using the *Limulus amoebocyte* lysate (LAL) test. In the field of environmental toxicity and pollution, the documents released by the US Environmental Protection Agency (EPA) seem to be more pragmatic (Table 3).

Nanosafety is one of the main fields of interest of European Commission as well. It released opinions, regulatory reviews, and working papers on this argument during the period 2007–2012, directly or through its departments, scientific committees, and agencies (Table 4). The synergic work of the laboratory of Nanobiotechnology with the European Union Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM), the laboratory of *In Vitro* Methods (IVM), and that of Predictive Toxicology, all belonging to the Institute for Health

and Consumer Protection (IHCP), significantly improved the knowledge in the field. A significant number of publications are accessible from their archives (http://ihcp.jrc.ec.europa.eu/our_labs/predictive_toxicology/publications/articles and http://ihcp.jrc.ec.europa.eu/our_labs/eurl-ecvam/archive-publications). The safety of nanotechnologies for foods and packages was a matter of interest for the US FDA, which dedicated to this argument two presentations at the FDA Regulatory Meeting held in 2008 (Raymond, 2008; Schultz, 2008) and for the European Food Safety Authority (EFSA, 2009, 2011), which published in 2013 a technical report that focused on the outcome of previously issued reports and opinions.

3 REFERENCE STANDARDS

Standard NMs firstly appeared in 2007, when the National Institute of Standards and Technology

Table 4. List of documents recently released by the European agencies for standardization, concerning the regulatory purposes for nanosciences and nanosafety.

Agency	Year	Document identification	Content
EU Commission	2007	http://ec.europa.eu/health/ph_risk/committees/04_scenihr/docs/scenihr_o_010.pdf	Opinion on the appropriateness of the risk assessment methodology in accordance with the technical guidance documents for new and existing substances for assessing the risk of nanomaterials
	2008	http://ec.europa.eu/nanotechnology/pdf/nanocode-rec_pe0894c_en.pdf	Commission recommendation of 07/02/2008, on a code of conduct for responsible nanosciences and nanotechnologies research
	2009	http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=SEC:2009:1468:FIN:EN:PDF	Nanosciences and nanotechnologies: an action plan for Europe 2005–2009 (Report 2007–2009)
	2010	http://dx.doi.org/10.2788/98686	Lövestam <i>et al.</i> (2010): Considerations on a definition of nanomaterial for regulatory purposes
	2011	http://dx.doi.org/10.2777/2209	De Victoria (2011): Successful European nanotechnology research, including sections discussing the safety issue
	2011	http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2011:275:0038:0040:EN:PDF	Commission recommendation of October 18, 2011, on the definition of nanomaterial (text with EEA relevance), <i>Official Journal of the European Union</i> , L275/38
	2012	http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=COM:2012:0572:FIN:en:PDF	Second regulatory review on nanomaterials (text with EEA relevance)
	2012	http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=SWD:2012:0288:FIN:EN:PDF	Types and uses of nanomaterials, including safety aspects (Commission Staff working paper accompanying the previous regulatory review)
	2010	http://ec.europa.eu/health/scientific_committees/emerging/docs/scenihr_o_032.pdf	Scientific basis for the definition of the term “nanomaterial”
EFSA	2009	http://dx.doi.org/10.2903/j.efsa.2009.958	The potential risks arising from nanoscience and nanotechnologies on food and feed safety
	2011	http://dx.doi.org/10.2903/j.efsa.2011.2140	Guidance on the risk assessment of the application of nanoscience and nanotechnologies in the food and feed chain
	2012	http://www.efsa.europa.eu/en/search/doc/362e.pdf	Annual report of the EFSA Scientific Network of Risk Assessment of Nanotechnologies in Food and Feed for 2012
	2013	http://www.efsa.europa.eu/en/search/doc/126e.pdf	Outcome of the public consultation on the draft scientific opinion on guidance on risk assessment concerning potential risks arising from applications of nanoscience and nanotechnologies to food and feed

SCENIHR, Scientific Committee on Emerging and Newly Identified Health Risks.

(NIST) approved polystyrene nanosphere, 100 and 60 nm, followed by gold NPs (10–30 and 60 nm), single-walled carbon nanotubes (SWCNTs), and TiO₂ NPs (sold as micro-sized aggregates, sonication deliver particles sized 70–77 nm). Polystyrene and gold NPs are classified as nontoxic and non-carcinogenic, whereas TiO₂ NPs and SWCNTs are rated as irritant. TiO₂ NPs are also classified as a possible carcinogen by inhalation. Other details are shown in Table 5 and in the NIST US web site.

Thanks to their regular shape and easy synthesis, the polystyrene NPs were exploited for the development of new methods for nanometrology, computational biology, pharmacology, and toxicology, as exemplified in Table 6. The effects of the functionalization at the surface were tested (De Sousa Delgado, Leonard, and Dellacherie, 2000; Gurevich *et al.*, 2011; Lundqvist *et al.*, 2008; Sant and Gale, 2012; Zhang *et al.*, 2011). These NPs are generally considered to be relatively inert. Nevertheless, some side effects were observed,

Table 5. Standard nanomaterials approved by the NIST (USA) and available at the NIST web site for sale.

Institute	Certificate date	Standard	Material	Size	Toxicity
NIST	01/10/2007	SRM 1963a	Polystyrene spheres	100 nm	Nonhazardous
NIST	01/10/2007	SRM1964	Polystyrene spheres	60 nm	Nonhazardous
NIST	12/13/2007	SRM 8011	Gold nanoparticles	10 nm	Skin and eye irritant, avoid ingestion
NIST	12/13/2007	SRM 8012	Gold nanoparticles	30 nm	Skin and eye irritant, avoid ingestion
NIST	12/13/2007	SRM 8013	Gold nanoparticles	60 nm	Skin and eye irritant, avoid ingestion
NIST	11/14/2011	SRM 2483	SWCNTs	Raw soot	Respiratory hazard
NIST	06/14/2012	SRM 1898	TiO ₂	70 nm	Respiratory hazard (cancerogenicity)

such as alteration of coagulation and iron uptake by the intestinal epithelium (Mahler *et al.*, 2012; McGuinness *et al.*, 2011). Embryotoxicity was described by Manabe, Tatarazako, and Kinoshita (2011) in model fish organism.

A repository of significant NMs, such as nanosilver, nanoclays, carbon nanotubes (CNTs), and different metal oxides, is available at IHCP since February 2011 (http://ihcp.jrc.ec.europa.eu/our_activities/nanotechnology/nanomaterials-repository). This collection is intended to support collaborative international studies with supply of common materials produced under Good Laboratory Practice.

4 ALTERNATIVE TESTS IMPLEMENTED FOR NANOTOXICITY STUDIES

In vivo toxicity tests in mammals are limited by economical, ethical, and practical considerations; therefore, “alternative” models are recommended. The International Co-operation on Alternative Test Methods (ICATM) is a network of agencies that coordinate the process of development and validation of these methods in a worldwide dimension. The European member is the EURL ECVAM, which maintains a database of validated methods, continuing the mission of the ECVAM, operating since 1991. Other members of the ICATM are the NICEATM/ICCVAM (National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods and Interagency Co-ordinating Committee on the Validation of Alternative Methods) operating in the United States, the ICATAM (Canadian International Co-operation on Alternative Test Methods), the JaCVAM (Japanese Center for the Validation of Alternative Methods), and the KoCVAM (Korean Center for the Validation of Alternative Methods). Alternative tests include *in vitro* models, performed in acellular

systems, in culture of isolated cell or cell lineages (both prokaryotes and eukaryotes), in isolated tissues maintained or grown *in vitro*, or in lower vertebrates. Test in mammals are classified as “alternative” if poorly invasive. These procedures seemed to be appropriate, with modifications, for the study of nanotoxicity (Hartung and Sabbioni, 2011; Clift, Gehr, and Rothen-Rutishauser, 2011). On the basis of the analysis of most recently issued experimental works, Figure 4 shows a possible decision tree for assessing nanotoxicity *in vitro*.

4.1 Descriptors of Toxicity in Acellular Systems

The acellular systems, such as media and other aqueous phases in which NMs are suspended, provided a very useful environment to characterize and study the colloid stability of NMs suspended in complex media (Lv *et al.*, 2012; Lowry *et al.*, 2012). Size, shape, aggregation and dissolution rate, surface charge and reactivity, magnetic properties, electron spin, and formation of oxygen-reactive species were parameters evaluated in acellular media (Al-Hallak *et al.*, 2010; Cohen *et al.*, 2013; Vippola *et al.*, 2009; Stone, Johnston, and Schins, 2009). The addition of proteins and other molecules acting as surfactants affects the ability of NMs to form stable suspensions, to stick together or adhere to other compounds. Consequently, the interactions with living systems could change and toxic properties develop and increase, or, instead, decrease and disappear.

The use and measure of predictors for chemical toxicity is complicated, in the case of NMs, by their still poorly defined chemistry and uniqueness of behavior. Tables 7 and 8 show some variations of the values of two popular chemical descriptors, the acid dissociation constant (pK_a) and the octanol–water partition coefficient ($\log P$ or $\log K_{ow}$).

Table 6. Polystyrene NPs exploited as standard material for developing methods or as inert standard in comparison with other nanomaterials.

Size, functionalization	Aim	Method	Reference
30–100 nm	Nanometrology	Sizing by angular dependence of DLS	Takahashi, Kato, and Kinugasa (2011)
100 nm	Nanometrology	Sizing by NTA for monodisperse NPs. Physical model developed to minimize Brownian motions	Saveyn <i>et al.</i> (2010)
30 and 100 nm, fluorescently labeled	Nanometrology	Zeta-potential: μ -EFFF	Chang, Dosev, and Kennedy (2007)
260 nm	Nanometrology, immunogenicity	Size, electrophoretic mobility, colloidal stability, immunoreactivity of the immobilized IgG Compared with PLGA NPs (210 nm) coated with IgG	Santander-Ortega, Bastos-Gonzalez, and Ortega-Vinuesa (2007)
130–210 nm, uncoated versus carboxylated	Fractionation	New microscale ThElFFF channel	Sant and Gale (2012)
20, 50, 93, and 135 nm	Fractionation	Asymmetrical flow field-flow fractionation channels. Profiles: exponential > trapezoidal $\gg$ rectangular	Ahn <i>et al.</i> (2010)
200 nm, sulfate	Concentration measurement	Gold positively charged sensor surface	Gurevich <i>et al.</i> (2011)
50 and 100 nm, unmodified, aminated, or carboxylated	Pharmacology	<i>In vitro</i> acellular systems: characterization of the corona	Lundqvist <i>et al.</i> (2008) Zhang <i>et al.</i> (2011)
57 nm	Pharmacology	<i>In vitro</i> acellular systems: agglomeration in DMEM Comparison with <i>n</i> Au (30 nm), <i>n</i> Ag (23 nm), <i>n</i> CeO ₂ (64 nm)	Zook <i>et al.</i> (2011)
200 nm (unmodified), 295 nm (coated with vitE), 260 nm (coated PGLA)	Pharmacology	<i>In vitro</i> cellular systems: uptake by Caco-2: 295 > 260 > 200 nm	Win and Feng (2005)
50 nm to 6 μ m, uncoated, PLGA, PEG/PLGA, chitosan/PLGA	Pharmacology	<i>In vitro</i> trachea: mucociliary clearance assessment by size, zeta-potential, and functionalization PEG/PLGA > PLGA = uncoated = chitosan-PLGA	Henning <i>et al.</i> (2010)
10–100 nm	Computational biology	<i>In silico</i> kinetics simulation of the size-dependent kinetics and deposition pattern in human lung after nasal inhalation	Dong <i>et al.</i> (2010)
20–1100 nm	Computational biology: ISDD	S/O ET: 20 nm: 0.95; 100 nm: 0.51; 1100 nm: 0.37 Compared with amorphous silica (35 nm) and iron oxide (30 nm)	Hinderliter <i>et al.</i> (2010)

DLS, dynamic light scattering; DMEM, Dulbecco's modified Eagle medium; HIV, human immunodeficiency virus; ISDD, *in vitro* sedimentation, diffusion, and dosimetry; μ -EFFF, microelectrical field-flow fractionation; NTA, nanoparticles tracking analysis; PLGA, poly(lactic-co-glycolic acid); PVA, polyvinyl alcohol; S/O ET, simulated/observed ratio for endo-cellular transport; ThElFFF, thermal-electrical field-flow fractionation (ThElFFF).

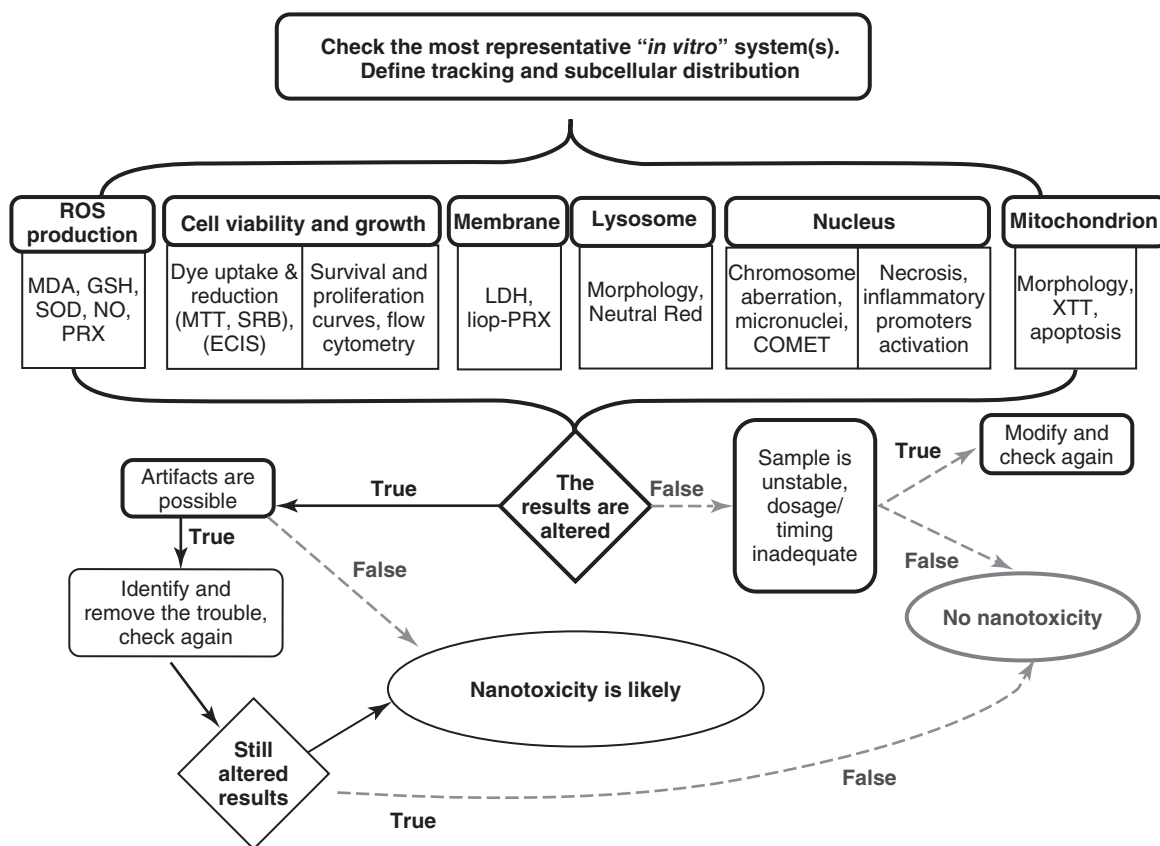


Figure 4. A decision tree for the assessment of cytotoxicity of NMs “*in vitro*.” Decisional steps are inserted in boxes with rounded angles, executive steps are in rectangles, the output is in rombic icons, and the final conclusion is in oval icons. COMET, single-cell gel electrophoresis assay; ECIS, electric cell-substrate impedance sensing; GSH, glutathione, reduced form; LDH, lactate dehydrogenase; MDA, malonyl dihaldeyde; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; NO, nitric oxide; PRX, peroxidase; ROS, reactive oxygen species; SOD, superoxide dismutase; SRB, sulforhodamine B; XTT, 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide.

In nanochemistry, they are related to a number of variables not strictly dependent on the chemical nature of the compound. The pK_a plays a role in NP aggregation and partial dissolution processes; its values are sensitive to the coating and functionalization of the surface, as well to the shape and size of NPs. The curvature of the NP's surface modifies the pK_a by altering the charge distribution (Wang *et al.*, 2011). The assessment of the pK_a values of NMs is somewhat similar to that of folded proteins and, as such, it was resolved experimentally (Ivanov, 2011) and computationally (Simonson, Carlsson, and Case, 2004; Li, Robertson, and Jensen, 2005).

The $\text{Log}P$ describes the hydrophobicity of chemicals and is included among the predictors of the hazard for aquatic toxicity and the oral bioavailability of chemicals (Papa, Villa, and Gramatica, 2005; Parrott *et al.*, 2005; Veber *et al.*, 2002). This parameter was found to be a descriptor of artificial lipid bilayer and liposomes – two strictly related nanostructures. The artificial lipid bilayer, structurally a nanosheet, is an acellular system with higher structural complexity than buffered solution, culture media, or blood plasma. Its dynamics was described at the nanoscale (Spurlin and Gewirth, 2007a). Two-dimensional (2D) maps of the areas accessible to the solvent at the surface

Table 7. Assessment of nanotoxicity *in vitro*: the pK_a as an example of chemical descriptors obtained in acellular systems.

Nanomaterial	Size (nm)	Shape	pK_a	Year and doi
CdS, coated with MAA	4.5	Q-dots	$pK_a(\text{COOH}) = 3.68$ (sol), 2.2 (ads)	Young, Green, and McQuillan (2006)
CdS, coated with MPA			$pK_a(\text{COOH}) = 4.3$ (sol), 3.8 (ads)	
CdTe, GSH capped: QD550/QD600	30	Q-dots	QD550: $pK_a = 5.2$; QD600: $pK_a = 6.3$	
Gold NPs assembled to I-motifs (ssDNA, C-rich domains)		Sphere	Assembly at $\text{pH} < pK_a = 6.5$, reversible at $\text{pH} \geq 6.5$	Wang <i>et al.</i> (2007)
Gold NPs, coated with PSS	5	Sphere	Stabilization at $\text{Ph} = 10$ ($>pK_a$ of DMAP)	Dorris <i>et al.</i> (2008)
Gold NPs, adsorbed on silica; layer of ToA	15	Sphere	$pK_a = 5.62$; pK_a (ToA sol) = 4.76	Rooth and Shaw (2006)
Si (ORMOSIL) plus dyes	33		Variation of pK_a at the interface $pK_a \cong 6.4$	
PEG/dipropylenetriamine, diamine side chain	~ 100	Micelle	Primary amine $pK_a \cong 9.9$ Secondary amine $\cong 6.4$	Itaka <i>et al.</i> (2004)
PEG-polycation, diamine side chain		Micelle	Primary amine $\cong 10$ Secondary amine $\cong 6.5$	Kataoka <i>et al.</i> (2005)
Amphiphilic PAA-b-PpHS	70–25, pH dependent	Micelle	$pK_a = 4$ (PAA), $pK_a = 10$ (PpHS)	Lee <i>et al.</i> (2008)
Amphiphilic azobenzene dye 1		Micelle	NPs assembly at $\text{pH} = 3\text{--}9$ $pK_a = 9.38$; aggregation at $\text{pH} > pK_a$	
CSO-SA, cationic		Micelle	NH_2 : [C] 9.79–63.41% $pK_a = 8.16\text{--}6.02$; aggregation stable at higher pK_a	Du <i>et al.</i> (2010)
α -CHCA		Dendron, G1–G3	G1/G2/G3: $pK_a = 4.4/5.6/6.2$ T/W = 3.3/0.52/0.53	Gomez-Escudero <i>et al.</i> (2008)
PAMAM G3 plus triclosan		Dendron	Triclosan soluble at $\text{pH} > pK_a = 8.4$	Gardiner <i>et al.</i> (2008)
PAMAM-NH ₂		Dendron, G4	G4: $pK_a = 9.52$ regulates the interaction on functionalized gold probes	

α -CHCA, α -cyano-hydroxycinnamic acid; CSO-SA, stearic acid-grafted chitosan oligosaccharide; DMAP, dimethylaminopyridine, stabilizer; G1–G3, dendrimers, from first to third generation; GSH, glutathione; MAA, mercaptoacetic acid; MPA, mercaptopropionic acid; PAA-b-PpHS, poly(acrylic acid)-b-poly(*p*-hydroxystyrene); PEGLL-PLL, poly(diethylene glycol-L-lysine)-poly(L-lysine); ToA, thioctic acid; T/W, toluene/water partition coefficient.

were constructed on the basis of the local $\text{Log}P$ distribution, expressing the spatial molecular hydrophobicity potential (Pyrkova *et al.*, 2011). Lipid bilayer interacting with NMs shows changes in phase transition, which accompany the adsorption of NMs to the surface, or penetration into the lipid tails, of the particles (Goertz *et al.*, 2011; Mecke *et al.*, 2005; Sholto and Ehrenberg, 2008; Spurlin and Gewirth, 2007b). Lipid layers could be considered as the precursors of liposomes, whose ability to capture drugs and release them, in strict dependence from environmental changes, is related to the $\text{Log}P$ values (Cern *et al.*, 2011).

4.2 In Vitro Toxicity Test: Cultured Cell

The tests performed *in vitro* in cellular systems represent the largest part of all studies on the toxicity of engineered NMs. Those assessing nanotoxicity in prokaryotes are usually focused on environmental issues (Handy *et al.*, 2012b) or on the development of new antibiotics, antivirals, and pesticides. These fields will not be considered in the context of this review. We will not consider as well the great deal of studies on cytotoxicity in cancer cell lines, aimed for the development of anticancer agents.

The development of complex protocols to assess nanotoxicity in eukaryotes was recently reviewed

Table 8. Assessment of nanotoxicity *in vitro*: the octanol–water partition coefficient ($\text{Log}K_{\text{o/w}}$ or $\text{Log}P$), as an example of chemical descriptor for environmental toxicity, obtained in acellular systems.

Nanomaterial	Size (nm)	$\text{Log}K_{\text{o/w}}$ ($\text{Log}P$)	Reference
C60		6.67	Jafvert and Kulkarni (2008)
Purified MWCNTs	30–70 × 386	$\text{Log}K_{\text{tw}} = 8.44$; $\text{Log}S = 1.11 - 10^{-11}$ (7.96 ng/l) 2.95 after 4 h sonication; ND after 8 or 16 h	Petersen, Huang, and Weber (2010)
Acidic (3 : 1) MWCNTs	30–70 × 407	2.77 after 4 h sonication; 2.69 after 16 h	Petersen, Huang, and Weber (2010)
SWCNT (according to chirality: m,n)		$\text{Log}P = -3.9193 + 3.7703n - 3.6001m$ $\text{Log}S = -5.1041 - 3.5075n - 3.5941m$	
Aqueous C60	87	3.08 ($K_{\text{a,o/w}}$)	Xiao and Wiesner (2012)
THF C60	55	2.54 ($K_{\text{a,o/w}}$)	
Fullerol	98	0.12 ($K_{\text{a,o/w}}$)	
Au–CIT	31	0.38 ($K_{\text{a,o/w}}$)	
Ag–CIT	52	0.03 ($K_{\text{a,o/w}}$)	
Ag–PVP	48	2.26 ($K_{\text{a,o/w}}$)	
Ag–GA	16	2.14 ($K_{\text{a,o/w}}$)	
PAMAM dendrimer 1, G4		G4 = -2.38 G0 = -1.53 ; G1 = -1.81 ; G2 = -1.99 ; G3 = -2.24 ; G5 = -2.16 ; G6, G7 = ND	Giri <i>et al.</i> (2009)
PAMAM dendrimer 2, 4–11G4		D2: -2.47 ; D4: -2.53 ; D5: -1.39 ; D6: -2.45 ; D7: -2.54 ; D8: -2.35 ; D9: -2.25 ; D10: -2.33 ; D11: -2.62	
PAMAM dendrimer 3, G3.5		-2.33	
PAMAM dendrimers		$K_{\text{o/w}} = \{[\Delta G_{\text{w}}^{\text{el}}(i) + \gamma_{\text{w}}A_{\text{w}}(i)] - [\Delta G_{\text{o}}^{\text{el}}(i) + \gamma_{\text{o}}A_{\text{o}}(i)]\} / 2.30RT$	

M/SWCNT, multi-/single walled carbon nanotubes; THF, tetrahydrofuran; CIT, citrate; PVP, polyvinylpyrrolidone; GA, gum Arabic; PAMAM dendrimer 1–7, the core is ethylene diamine (EDA); the terminal groups vary (NH_2 , amidoethanol, sodium carboxylate, succinamic acid, tris (hydroxymethyl) aminomethane, pyrrolidinone, amidoethylethanolamine, respectively). PAMAM dendrimers 8–11, the terminal group is NH_2 , the core is DAB (diaminobutane), DAH (diaminohexane), DAD (diaminododecane), and cyst (cystamine), respectively. The pH is 7.4, room temperature. G0–G7: dendrimer generation. $K_{\text{a,o/w}}$: see Giri *et al.* (2009).

(Arora, Rajwade, and Paknikar, 2012; Blank, Gehr, and Rothen-Rutishauser, 2009; Cattaneo *et al.*, 2010; Gornati *et al.*, 2009; Iavicoli *et al.*, 2011; Jeong *et al.*, 2009; Kroll *et al.*, 2009; Ma, 2009; Nagai and Toyokuni, 2010; Schrand *et al.*, 2012; Sharifi *et al.*, 2012; Yu *et al.*, 2009). The validated strategies and methods can, however, be applied to nanotoxicology only after careful evaluation and needed modification, to avoid false results due to unpredictable interferences between NMs and reagents or dyes (Hamid *et al.*, 2004; Ciofani *et al.*, 2010; Coccini *et al.*, 2010; Davis *et al.*, 2007; Hsiao and Huang, 2011; Kong *et al.*, 2011). The presence in the test system of toxic bulk materials possibly derived from dissolution or contamination with residual of production must also be carefully considered, as recommended by a recent document issued by ISO (ISO 29701, 2010; Table 2). Main

toxic mechanisms at cellular level included morphological alteration of the cell, engulfment and damage of lysosomes (Ji *et al.*, 2012), arrest of the cell cycle (Han *et al.*, 2012), and activation of macrophages (Sato *et al.*, 2005). The development of reactive oxygen species (ROS) is common to a large number of engineered NMs, metals, metal oxides and transition metals (Bernardini *et al.*, 2011), fullerenes, and organic NPs. The oxidative stress often associated with the exposure to NMs could damage membranes, mitochondria, and nucleic acids. The death of the cell can be a direct consequence of the insult or a response to the activation of the mechanisms of apoptosis/autophagy and inflammation/necrosis. A complete protocol for the nanotoxicity assessment should thus consider the dissolution rates of NMs, a preliminary characterization of the nanopharmacokinetics, the permeability of different type of

cells, the tracking inside the cells, and the targeting at subcellular level (Weissig and D'Souza, 2010). The bioavailability, and the following cytotoxicity, not only depends on the nature of NMs themselves but on the cell type too. A strategic approach should mimic *in vitro* different ways to enter the organism, using representative cell lines. Respiratory epithelia, gastrointestinal cells lines, dermal fibroblasts, endothelia, and cells derived from the central nervous systems and the BBB have been turned into systems suitable for nanotoxicological studies. Highly standardized cellular systems are the immortalized murine fibroblasts Balb/c 3T3 (Papis *et al.*, 2007; Ponti *et al.*, 2009), the colon carcinoma (Caco-2) cell (Castillo-Garit *et al.*, 2007; Duncan and Izzo, 2005) to test the intestinal adsorption of drugs and the A549 cell, a model for the AT II lung alveolar epithelial cell (Yang *et al.*, 2012).

4.3 *In Vitro* Toxicity Test: Isolated and Cultured Tissues

Isolated tissues represent a relatively old but recently revisited approach to test toxicity *in vitro*. Systems such as slices of lung tissues and isolated skin in a flow-through diffusion cell were used to test local toxicity (Baynes, 2001; Dong *et al.*, 2011). An elegant and recent modification of this type of approach was represented using three-dimensional (3D) cell cultures and microorgans developed *in vitro*. Different types of cells, mainly stem cells, develop into layered gems of 3D tissue, when harvested on regularly organized scaffolds. The NIST recommended this method, in particular the use of 3D hydrogel scaffolds as a convenient environment for growing cells, testing short- and medium-term effects of exposure to NPs and changes in NP properties (Mansfield *et al.*, 2013). Nanostructured hydrogels, fibers, and porous materials represent in fact favorable environments (Liu and Zhao, 2011; Schindler *et al.*, 2006; Yamada and Cukierman, 2007), in which very complex systems, such as beating heart, nasal mucosa with ciliated epithelium, and two-cellular model of the BBB have been reproduced (Hackenberg *et al.*, 2011; Ou *et al.*, 2011; Sansing, Renner, and MacLean, 2012). In comparison with corresponding 2D cell cultures, the 3D ones allow a more consistent evaluation of the bioavailability in the internal layer and a more realistic model of pharmacodynamics. The SWCNTs,

which showed some toxicity in 2D cultures, seemed instead to be safe in 3D cultures (Movia *et al.*, 2011), whereas ZnO NPs were toxic to the human nasal mucosa miniorgan (Hackenberg *et al.*, 2011). In addition to their value for nanotoxicology studies (Child *et al.*, 2011; Kirsch-Volders *et al.*, 2011; Lee *et al.*, 2009; Ng and Pun, 2008), 3D scaffolds can also be implanted in humans and animals for the reconstruction of endangered tissues, such as the hydroxyapatite nanofibers, which stimulate the regrowth of bones after injuries (Nirmala *et al.*, 2012). Nanofibers organized in 3D net maintained pancreatic islets viable and functional (Zhao *et al.*, 2010) and supported olfactory ensheathing cells (Zhu *et al.*, 2011).

4.4 Testing Toxicity: The Blood and Its Derivatives

The human blood and its derivatives retain autonomous roles in toxicology. Blood is the only fluid tissue in the organisms, whose cells are evenly exposed to substances entering the circulation. In addition, the blood is easily and safely collected; it is the site of coagulation and hemolysis – two unique responses to chemical and environmental stimuli.

NPs dispersed in blood and plasma undergo a series of modifications. First of all, there is the formation of “corona,” a complex of proteins adhering to the NP surface. Its composition is defined by proteomic methods in the case of polystyrene and gold NPs (Lundqvist *et al.*, 2008; Walkey *et al.*, 2012; Zhang *et al.*, 2011). Shape, surface properties, and chemical nature of NPs are important factors in determining their affinity to several plasma proteins (Walkey and Chan, 2012). Fibrinogen, serum albumin, and immunoglobulins were actively studied (Ruh *et al.*, 2012), together with apolipoproteins, which have been shown to mediate the permeability of the BBB to NPs (Liu *et al.*, 2006; Ruh *et al.*, 2012).

The nature and dynamics of binding were clarified in the case of SWCNTs. The aromatic residues present in certain amino acids seemed to play a fundamental role by interfering with the π - π bonds of the fullerenes. This mechanism seemed to be protective against SWCNTs toxicity (Ge *et al.*, 2011).

Tiered protocols for assessing *in vitro* toxicity of NPs proposed a series of routine clinical tests, including the thrombogenic properties of the entire

blood, plasma, or platelet-rich plasma (Neun and Dobrovolskaia, 2011). Several different NPs displayed pro and antithrombogenic properties, such as polystyrene NPs functionalized at the surface (McGuinness *et al.*, 2011), cationic dendrimers (Jones *et al.*, 2012), amorphous nanosilica (Nabeshi *et al.*, 2012), metal oxides, and carbon-based NPs (Laloy *et al.*, 2012).

The red blood cells (RBCs) are another component of the blood largely used in clinical testing. Changes in shape, membrane fluidity and rheological properties, or leakage of hemoglobin, with various degrees of hemolysis and aggregation, represented their main reactions to toxic agents, including several NMs (Li *et al.*, 2008 (TiO₂); Mishra, Gupta, and Jain, 2010; Wang *et al.*, 2010; Qiao *et al.*, 2007). When interacting with the cell surface, the NMs induce a rapid redistribution of negative charges, affecting the ability of RBC to remain in suspension. Sublethally damaged RBCs show a tendency to aggregate, *in vitro* or *in vivo*, in the smallest capillaries. In several cases, the damage cannot be repaired, and it persists as long as the affected RBCs are circulating. Therefore, this system could represent a long-lasting marker of a short exposure to NMs with toxic effects. For more aggressive insults, the routine screening could take advantage from the hemolytic test, performed on the entire blood or on washed red cells. Potentially hemolytic NMs include TiO₂ NPs (Li *et al.*, 2008), cationic and anionic Au nanospheres of 30 nm (Love, Thompson, and Haynes, 2012), amine-modified graphene (Singh *et al.*, 2012), and uncoated dendrimers (Mishra, Gupta, and Jain, 2010; Wang *et al.*, 2010; Ziemba *et al.*, 2012).

The *in vitro* exposure to metallic NPs activated human mononuclear cells, a component of white blood cells, and induced a reaction similar to that seen in experimental and clinical autoimmunity (Petrarca *et al.*, 2006).

5 ALTERNATIVE MODELS TO TEST NANOTOXICITY IN MULTICELLULAR ORGANISMS

5.1 Ecotoxicity Assessment

The necessary platform to study the ecotoxicological impact of NMs on living organisms has rapidly evolved. The studies in this field were

almost always performed in invertebrates and lower vertebrates, alias the recommended “alternative” models. Aquatic pollution was tested in conventional models, such as *Daphnia* sp., or in more unusual organisms, invertebrates and fish. Soil pollution was instead tested on worms, snails, and terrestrial crustaceans, in addition to prokaryotes. Studies in plants were also considered (Cattaneo *et al.*, 2009; Gogos *et al.*, 2012; Handy *et al.*, 2012a, 2012b; Jovanović and Palić, 2012; Dietz and Herth, 2011; Miralles, Church, and Harris, 2012; Navarro *et al.*, 2008). The resulting knowledge, however, is limited by the simplified conditions that have been generally used in the hazard assessment. Instead, the NM interactions with the environment follow rather complex rules; therefore, the poor knowledge of fundamental parameters raises doubt on the adequacy of the present approach (Baun *et al.*, 2009; Christian *et al.*, 2008; Delay and Frimmel, 2012; Dong and Lo, 2013; Kasel *et al.*, 2013; Lin *et al.*, 2012a, 2012b; Meesters *et al.*, 2013; Scown, van Aerle, and Tyler, 2010).

5.2 Embryonic Toxicity Assessment: The Role of Invertebrate and Lower Vertebrate Models

The hazard for embryotoxicity and teratogenicity of NMs was mainly investigated in organisms belonging to “alternative models.” Among them, the fruit fly (*Drosophila melanogaster*), the African clawed frog (*Xenopus laevis*), and the zebrafish (*Danio rerio*) were preferred because of the availability of extensive and public databases (Fly Base, Xenbase, and ZIRC, respectively).

The genotoxicity of NMs was studied in communities of prokaryotes or in *Salmonella typhimurium*, with the commercially available AMES test, in cultured eukaryotic cells and in *D. melanogaster* (Doak *et al.*, 2012; Graf *et al.*, 1984; Lehmann *et al.*, 2000; Pfuhler *et al.*, 2013). Demir *et al.* (2011) and Vales *et al.* (2013) demonstrated that Ag and Co NPs mixed with food induced somatic recombination in third instar larvae, whereas silica NPs (ORMOSIL[®], 20 nm) accumulated mainly in the nervous tissue of first instar larvae without affecting further development (Barandeh *et al.*, 2012).

Aquatic invertebrates represent the largest group of organisms in which environmental and embryonic nanotoxicity was tested. The side effects of exposure

to several types of NPs were studied in arthropods (*Daphnia magna*, *Thamnocephalus platyurus*, *Chironomus riparius*, and *Artemia salina*), molluscs (*Haliotis diversicolor supertexta*, *Crassostrea virginica*, and *Mytilus galloprovincialis*) and echinoderms (*Psammechinus miliaris* and *Paracentrotus lividus*). This apparently disordered surplus of species reflects the biological diversity of organisms adapted to different aquatic habitats, with different modalities of feeding and freely moving or sessile. All these differences can influence the degree of exposure to the NMs dispersed in the environment. Therefore, customized or tiered and standardized toxicity tests have been developed in these species. Alternative models in invertebrates were discussed in a previous review (Cattaneo *et al.*, 2009). NPs of iron and iron oxide reduced the fertilizing potential of the sperm of *M. galloprovincialis*, *Ciona intestinalis*, and *P. miliaris* (Kadar *et al.*, 2010, 2013). C60 negatively affected the embryonic development of *C. virginica* (Ringwood, Levi Polyachenko, and Carroll, 2009); TiO₂ NPs were embryotoxic in *H. diversicolor supertexta* (Zhu *et al.*, 2009). The delayed/disrupted embryonic development seemed to be species specific, with *P. miliaris* being the most resistant organism (Kadar *et al.*, 2013). Cationic solid lipid NPs, designed to carry RNA, did not affect the viability of embryonic *P. lividus* (Montana *et al.*, 2007). Lee, Kim, and Choi (2009) assessed the geno- and embryotoxicity of metal oxide NPs, other than iron, in freshwater arthropods. Both TiO₂ and CeO₂ NPs increased the mortality rate in adults and larvae and induced breaks of the DNA strands. The larvae showed growth retardation and lower reproduction potential once developed into a mature individual. The NPs mainly accumulated in the gut and the clearance was very low (Zhu, Chang, and Chen, 2010). The long-lasting accumulation, as well as the dose- and time-dependent toxicity, was recorded also in nauplii of *A. salina* (Ates *et al.*, 2013).

The teratogenicity and embryotoxicity of NMs were also tested with the *Frog Embryo Teratogenesis Assay in Xenopus* (FETAX), a powerful and flexible bioassay for developmental toxicants. It can be conveniently integrated with molecular techniques (Bernardini, Prati, and Gornati, 2005), and it can also be used for soil evaluation (Prati *et al.*, 2000). The higher effect, in terms of increased mortality rate and morphological changes after larval exposure to nanometals, was observed with ZnO NPs, whereas nano CuO was better tolerated

and TiO₂ and Fe₂O₃ NPs were safe (Nations *et al.*, 2011a, 2011b). TiO₂ NPs, however, enhanced the mortality rate when associated with UV, with a dose- and size-dependent effect (Zhang *et al.*, 2012). Well-characterized double-walled CNT, instead, did not show genotoxicity, evaluated by the micronucleus, nor enhanced mortality in *X. laevis* larvae (Mouchet *et al.*, 2008, 2011). The injection of quantum dots (QDs) by near-infrared laser was proposed as a method for selective ablation of cells in the embryos, on the basis of observation done in *X. laevis* (Umanzor-Alvarez *et al.*, 2011).

The third model organism, the zebrafish (*D. rerio*), is the most studied model for the embryotoxicity of NMs. Liu *et al.* (2012a) developed an automated method for the morphological analysis of the embryonic and early larval stage during exposure to NPs (and eventually other toxicants). The prototype allowed the simultaneous comparison of 96 wells, with three phenotype variants: hatched, unhatched, and dead. Each phenotype was coded and identified on the basis of the profile of opacity to the light of different regions of a microwall containing a single embryo. Assessed with these three parameters, the accuracy of the method was 97.14%. The authors claimed that the procedure was ready for the introduction of more refined variables. This test was specifically designed for NPs and tested with nanoparticulate silver and metal oxides. To summarize the results obtained with other NMs, buckyballs and CNTs, nanocrystalline cellulose, hydroxyapatite, and fluorescent core-shell silica seemed to be relatively safe, when no metal contaminants were present in the sample (Cheng, Flahaut, and Cheng, 2007; Fent *et al.*, 2010; Kovacs *et al.*, 2010; Usenko, Harper, and Tanguay, 2007; Xu *et al.*, 2012; Zhu *et al.*, 2007). Silica NPs caused some cardiotoxicity (Duan *et al.*, 2013), but much more aggressive against developing larvae were the QDs and the harmful ions resulting from their dissolution (George *et al.*, 2011; King-Heiden *et al.*, 2009). The embryotoxicity of nanosized transition metals and metal oxides seemed to be similar, or lower, than that of the corresponding bulk compounds, with the exception of the NPs of ZnO, whose toxicity was higher than that of *n*Ag and *n*TiO₂ (Asharani *et al.*, 2008; Bai *et al.*, 2010a, 2010b; Bar-Ilan *et al.*, 2009; Geffroy *et al.*, 2012; Krysanov and Demidova, 2012; Lee *et al.*, 2007; Lin *et al.*, 2011; Zhao *et al.*, 2013; Zhu *et al.*, 2008). Concurrent exposure to nano TiO₂ and light was toxic to the

Table 9. Common toxicological parameters, derived from the literature, concerning the effects of several NMs in the zebrafish model of embryonic and larval toxicity.

	NOEC (mg/l)	LOEC (mg/l)	IC (or LC) 50 (mg/l)	Max. effect dose (mg/l)
Mortality rate				
QD	1	5	1–15	15
ZnO	Not observed	15	25	Not observed
nAg	5	10	Not reached	10
nPt, nAu	Not observed	Not observed	Not observed	Not observed
nAl ₂ O ₃ , nSiO ₂	Not observed	Not observed	Not observed	Not observed
Hatching inhibition				
QD	1	5	5–15	15
ZnO	1	1–5	Not reached	5
nAg	5	5–15	<15	Not observed
nPt	5	15	Circa 25	Not observed
nAu	Not observed	Not observed	Not observed	Not observed

larvae of zebrafish, possibly as a consequence of the release of reactive species of oxygen (Bar-Ilan *et al.*, 2012). The toxicity of Fe₃O₄ NPs was low in the developing zebrafish, also when a more sensitive method was applied by anticipating the observation time to days 1–6 post-fertilization (Liu *et al.*, 2012b). Table 9 summarizes several toxicity parameters of NMs in the zebrafish.

The bioavailability and distribution of potentially inert polystyrene nano- and microparticles were studied in another model organism, the medaka, *Oryzias latipes* (Shima and Shimada, 1994). In the fertilized egg, embryos and adult medaka, particles spread according to their size. Larger microparticles (>500 nm) were generally safe and mainly accumulated in the oil droplets of egg, whereas the smaller ones (<500 nm) had the largest bioavailability in the egg but did not accumulate in the embryos. NPs (circa 40 nm) shifted instead into the yolk sac and in the gallbladder of developing embryos were slowly cleared and could reduce the survival of embryos. In adult fish, the NPs accumulated mainly in the gills and gut, but small amounts were also found in the brain, suggesting their ability to cross a fully developed BBB (Kashiwada, 2006; Manabe, Tatarazako, and Kinoshita, 2011). Ag NPs were more toxic to the medaka; they reached the chorion and persisted adherent to the surface or entered and damaged the membranes of the egg. Colloidal Ag delayed development and growth and induced irreversible side effects with nonlinear kinetics, dose- and time-dependent (Kashiwada *et al.*, 2012; Wu and Zhou, 2012). Markers of oxidative stress were higher after exposure (Kashiwada *et al.*, 2012; Li *et al.*, 2009 and 2012; Wu and Zhou, 2012;

Wu *et al.*, 2010). Genes involved in the response to oxygen stressors were affected in some of the observed malformations and strikingly activated (Kashiwada *et al.*, 2012). The toxicity of Ag NPs seemed, however, to be limited to the developing medaka; it was ineffective in adult fish (Kashiwada *et al.*, 2012; Li *et al.*, 2012; Wu *et al.*, 2010). TiO₂ NPs accumulated in the chorion of fertilized eggs and caused pericardial edema, delayed development, and premature hatching of embryos, without increased mortality. The posthatch mortality was increased at day 15. These effects were in general dose dependent (Paterson *et al.*, 2011). The exposure to fluorescent silica NPs had a negligible effect on the hatchability, but induced early abnormalities in eggs, possibly related with the colloidal instability of the NPs, because of the modalities of preparation (Lee *et al.*, 2011). In addition, for C60, which enhanced glutathione consumption, mortality, and malformations rate, the modalities of preparation determined the toxicity hazard. Aqueous suspensions were generally safer if stirring and addition of toluene and dimethyl sulfoxide (DMSO) were avoided (Kim *et al.*, 2010). Au NPs counteracted the positive effect of heparin sulfate on muscle development in chicken embryos, without increasing mortality or inducing malformations (Zielinska *et al.*, 2011).

5.3 Embryonic Toxicity Assessment: Studies in Placental Mammals

Placental barrier protects the fetal compartment from the potential aggression of substances present

in the maternal blood. NMs are potentially threatening substances because of their ability to cross highly selective biological barrier (See Chapter 13; Kanwar *et al.*, 2012; Zarbin *et al.*, 2012). The risk assessment for the fetuses required the exposure of pregnant mice and rats with fully developed placentas to NMs by different routes, inhalation or intravenous (i.v.) injection. The toxic effects observed in the offsprings depended on dose, size, chemistry, and functionalization of NPs. Among the tested NMs, we recall carbon and metal-based NPs. The effects, when observed, varied greatly, ranging from impaired placental and fetal growth to intrauterine mortality and liver underdevelopment. The crossing of the placenta by the NPs remained undemonstrated as they seemed to accumulate only at the maternal compartment of the placenta (Blum *et al.*, 2012; Hougaard *et al.*, 2010; Jackson *et al.*, 2012, 2013; Zalgevičienė *et al.*, 2012). Therefore, the effects of the treatment on the offsprings may be a consequence of the alterations of the maternal

health, although also some severely diseased mothers carried healthy fetuses (Jackson *et al.*, 2012; Hougaard *et al.*, 2010; Zalgevičienė *et al.*, 2012). Size and chemical nature seemed to influence the kinetics of NMs at the placental barrier (Yamashita *et al.*, 2011; Sumner *et al.*, 2010; Chu *et al.*, 2010).

Because of anatomical differences between their placentas, studies in rodents were considered to be poorly predictive. Therefore, Myllynen *et al.* (2008) proposed the perfused human placenta as a new model. Polystyrene NPs sized 50 nm reached the fetal compartment without affecting the viability and the morphology of the placenta (Wick *et al.*, 2010). The Au NPs were instead unable to completely cross the organ; they reached the maternal compartment only, and the polyethylene glycol (PEG) coating prevented even this partial penetration (Myllynen *et al.*, 2008). The polyamidoamine (PAMAM) dendrimers could completely cross the barrier, whereas their concentration was much lower

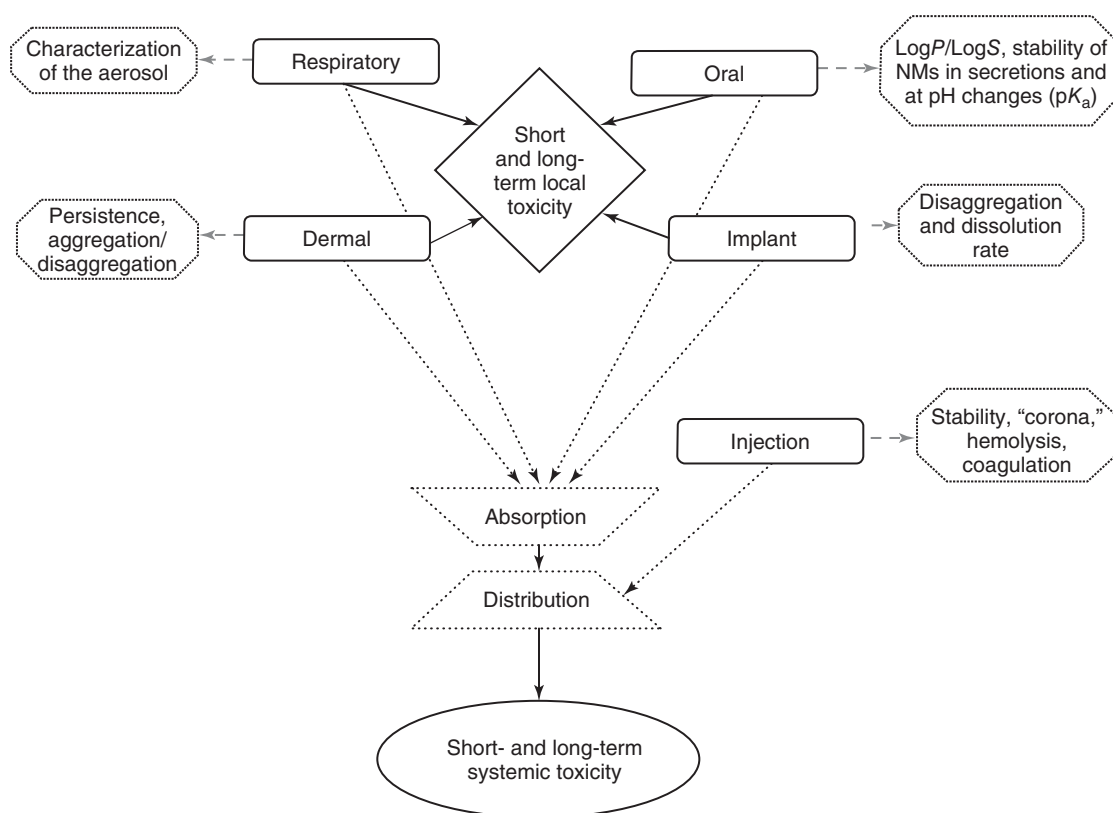


Figure 5. A decision tree for the assessment of cytotoxicity of NMs in “*in vivo*” studies. The routes of exposure are considered.

in the fluid collected at the fetal side. The fetal vacuolar space showed morphologic changes, affecting the fetal circulation (Menjoge *et al.*, 2011).

6 IN VIVO TOXICITY

The assessment of *in vivo* toxicity of NMs, especially those exploited for the use in humans and domestic animals, needs a description of their pharmacodynamics in terms of absorption, distribution, metabolism, excretion, and toxicity (ADMET) (Cao *et al.*, 2012; Hou *et al.*, 2009). Unfortunately, the standardization of studies on nanotoxicity of engineered NMs is still insufficient to produce descriptors robust enough to reach predictive strength. With several exceptions, data were mainly obtained in laboratory animals, and different ways of exposure were tested; Figure 5 displays different modalities of impact of NMs with the body and summarizes main descriptors of toxicity hazard. In addition to rodents, fish have been increasingly studied after exposure to NMs. Despite the poorly comparable modalities of exposure in fish and mammals, fish are interesting organisms not only as models for aquatic toxicity but also as an important component of food chains. Following the veterinary use or the accidental dispersion in the water, the NMs could eventually act as toxic chemicals and/or accumulate in the edible tissues of the fish, representing a potential threat for predators and humans. This fact confers a more striking interest to the increasing number of experimental works in nanotoxicology applied to species of fish relevant for human nutrition and aquaculture (Al-Bairuty *et al.*, 2013; Boyle *et al.*, 2013; Cedervall *et al.*, 2012; Gagné *et al.*, 2012; Shaw, Al-Bairuty, and Handy, 2012).

6.1 Systemic and Local Toxicity After Inhalation

Among hazardous airborne pollutants, ultrafine particles in diesel exhaust, smokes of combustion, and asbestos are long since known. Their dimension, often at the nanoscale, posed a concern about safety of engineered particles, by analogy. Notwithstanding that only very few reports described acute effects in humans exposed to engineered NMs, and that chronic effects have never been reported until now,

the alert remains high. Accumulation in lower airways could lead to permanent lung damage, whereas NPs persisting in nose and pharynx can eventually reach the brain, following the olfactory nerves (Chuang *et al.*, 2011; Donaldson and Seaton, 2012; Johnston *et al.*, 2013; Koivisto *et al.*, 2012; Nemmar *et al.*, 2012; Oberdörster, Stone, and Donaldson, 2007; Palomäki *et al.*, 2011; Sharma, 2010; Shvedova *et al.*, 2012; Simkó and Mattsson, 2010; Wang *et al.*, 2013). Owing to anatomical differences, the alternative models in this case are inadequate, and specially designed inhalation exposure chambers are available, for experimental use in rats or for clinical use in humans, which reproduce at the best the environmental exposure. The characterization of inhaled NPs in this type of chambers was the subject of a recently issued standard protocol (EN ISO 10808:2010). Studies in rodents, exposed to NMs by inhalation of aerosol or by intratracheal instillation, suggested that the clearance from the pulmonary fluids of different types of NPs is rapid, in part from ingestion, mostly through absorption in lymph and blood. The NPs persistently present in bronchoalveolar lavage were mainly found inside macrophages (Semmler-Behnke *et al.*, 2007). Deposition in the interstitial pulmonary space (Abid *et al.*, 2013; Ingle *et al.*, 2013; Mohammad *et al.*, 2013; Morfeld *et al.*, 2012) and local or systemic toxicity were described as well (Duffin, Mills, and Donaldson, 2007). The total deposition of particles ranging from 1 to 0.001 μm in human airways is described by typical U-shaped pattern, substantially confirmed by different computational models (Hofmann, 2011). However, according to Carvalho, Peters, and Williams (2011), the distribution of airborne NPs along the upper and lower airways should be redesigned under the light of recent advancement in imaging and detection methods. The dynamics, persistence, and clearance mechanisms of NPs from the lung are important not only for toxicological studies but also for the rational design of medicinal aerosols.

6.2 Systemic and Local Toxicity After Ingestion

Exposure by ingestion is common for a large number of compounds. In addition to oral formulations or foods containing NMs, ingestion could occur by chance, through partial swallowing of inhaled NMs,

or it can eventually originate from water contamination, dissolution from dental implants, residuals of food packages and toothpaste. The first level of toxicity is generally represented by acute reactions, mainly inflammatory or apoptotic, in the mucosa or in the surroundings layers. As the intestinal barrier generally limits the systemic bioavailability of ingested NMs, local toxicity was in many cases the only effect registered. Poorly absorbable NMs accumulate in the intestinal epithelium, and toxicity could follow the dissolution of toxic components. Once the intestinal barrier is crossed, the way of excretion of NPs and their dissolution products, biliary or urinary, was the most important determinant of the toxicity, targeted to the liver or kidney. Mutagenicity and carcinogenicity were also described (Sharifi *et al.*, 2012).

6.3 Systemic and Local Toxicity After Parenteral Administration

The parenteral route of administration is generally associated with the highest degree of bioavailability. However, in the case of NMs, bioavailability greatly depends on their characteristics. In the bloodstream, the rate of sequestration by macrophage is very rapid. This can be useful if the desired effect is to accumulate NPs at the site of inflammation. If not, NPs have to be modified at their surface to protect them from clearance. The most widely applied and well-standardized method is to add PEG at the surface, a technology known by the name STHEALTH® (Amoozgar and Yeo, 2012).

Once in the bloodstream, NPs mainly target liver, kidney, and heart. In Japanese medaka, the exposure to silver NPs was associated with enhanced activity of cytochrome P450, the main liver enzyme for processing drugs and chemicals (Chae *et al.*, 2009). In rodents, the parenteral administration of fullerenol, MWCNTs, and polymeric NPs altered the expression of P450 and its dependent enzymes (Ueng *et al.*, 1997; Hassan, El-Banna, and Elhusseiny, 2012; Ji *et al.*, 2009).

6.4 Systemic and Local Toxicity after Dermal and Ocular Application

One case report of severe skin reaction during professional exposure to dendrimers was reported

in recent years (Toyama *et al.*, 2008); notwithstanding, NMs of new generation have been conceived to be applied to the skin. The potential toxicity after administration through this route remains unexplored for many types of NMs (Papakostas *et al.*, 2011). Nanoformulations of Ag and TiO₂ are the active components of cosmetic products and textiles. Bandages and coatings containing silver NPs perform well in the treatment of long-lasting pressure wounds, burns, and ischemic lesions (Papakostas *et al.*, 2011). Similarly, the use of eye drops containing NPs has been claimed to be safe, better performing than traditional formulations, and able to reach anatomically protected regions of the eye (Zarbin *et al.*, 2012).

6.5 Toxicity of Implants

The knowledge about the ability of nanofibers to improve bone deposition was recently exploited for the construction of biocompatible implants. Nanoparticulate filling materials for dentistry were also introduced. Studies are still ongoing to define the hazard supported by their long-lasting persistence inside the body and the risk of dissolution (Polyzois *et al.*, 2012; García-Contreras *et al.*, 2011). Cattaneo *et al.* (forthcoming, See Chapter 13) discussed the hazard of acute and chronic toxicity of steroid-releasing intravitreal implants of nanoporous silica.

7 A COMPREHENSIVE APPROACH: METABOLOMICS

The term metabonomics was firstly introduced by Nicholson, Lindon, and Holmes (1999) and referred to the study of biochemical profiles expressing the differences in geno- and phenotypes, or the dynamic changes in response to nutritional, chemical, and physical stimulations. The near synonymous “metabolomics,” initially restricted to the use of this method in humans, is now prevalent. The procedure requires highly sophisticated technology (mainly nuclear magnetic resonance (NMR) and mass spectrometry) and skilled technicians, but it is regarded as advantageous in comparison with more traditional techniques because it provides an optimal platform for integrated studies in system biology, which spares time and money, assuming

that the basic requisites are acquired by the laboratory. Profiling metabolomics *in vivo* is minimally invasive; blood and urine can be the only required samples needed for a comprehensive description of different pathways. Other samples, such as cerebrospinal fluid, are needed for special purposes. Metabolomics became recently recognized in the “omics” sciences, and the Division of Systems Biology of the American Food and Drug Administration (FDA) included this approach, together with genomics and proteomics, to provide noninvasive and newer biomarkers of toxicity (Beger, Sun, and Schnackenberg, 2010; Schnackenberg and Beger, 2007).

This powerful tool will be of value in nanotoxicology or translational nanomedicine to speed up knowledge and routine follow-up, in a field with theoretical and technical difficulties (Duarte, 2011; Fowler, Conner, and Yamauchi, 2008; Sakamoto *et al.*, 2010; Schnackenberg, Sun, and Beger, 2012). Experimental researches in this field move their first steps and seem to validate the impression of suitability of metabolomics to study the impact of NMs *in vitro* and *in vivo*.

McNamara *et al.* (2011) compared the metabolomic profiles of mesenchymal stem cells cultured on common plastic substrates and that of the same cells grown on the nanofeatured surface of the TiO₂ implants, designed to stimulate the bone growth. The significant differences observed suggest that testing toxicity on plastic walls could be inadequate. Studies *in vivo* are also in an early phase. Metabolic profiles of multiple organ functions were simultaneously measured in rats after i.v. injection of ultrasmall superparamagnetic iron oxides (USPIOs) (Feng *et al.*, 2011) and silica NPs (Lu *et al.*, 2011, 2013), whereas TiO₂ Ag NPs were tested after oral and intratracheal administration (Bu *et al.*, 2010; Hadrup *et al.*, 2012; Tang *et al.*, 2010). The excretion of urinary metabolites of the Krebs's cycle was reduced in pregnant and lactating dams and rats, after i.v. injection of C60 (Sumner *et al.*, 2010). The results obtained until now with this new method seem to be of great interest in revealing new biomarkers and in giving new insights on the bioavailability, pharmacodynamics, and toxicity of NMs.

8 COMPUTATIONAL METHODS AND IN SILICO PREDICTION OF TOXICITY

To reach reliable predictivity, computational methods require large data sets with high level of standardization and reproducibility; these requirements are difficult to obtain in the field of nanotoxicity, and new theoretical approaches are necessary. Nevertheless, the way is now open and interest rapidly increases. In fact, several algorithms, mainly based on quantum chemistry, have been developed to calculate the most useful descriptors.

Carbon-based NMs are doubtlessly the most studied. Thanks to their apolarity and action as ion stabilizers; CNTs with narrow diameter (nanoneedles) can be able to release molecules through nonpolar biological media. According to what was predicted by quantum chemistry, at infinite length, these NMs acquire partial semiconductor properties (Poater *et al.*, 2009, 2012). Torrens (2005) and Petrova *et al.* (2011) successfully predicted the LogP, the LogS and the related value of Gibbs free energy of solvation (ΔG_{solv}) for C60 and SWCNTs with different chiralities. Another important descriptor of toxicity and genotoxicity is aromaticity, whose evaluation is complicated in NMs by the high degree of electron delocalization. The gauge including magnetically induced currents (GIMICs) method has been applied to evaluate the aromatic properties of different nanosystems, such as hydrocarbon nanorings and C60 fullerene (Johansson, Jusélius, and Sundholm, 2005; Taubert *et al.*, 2008, 2009). Raffaini and Ganazzoli (2007, 2010) simulated *in silico* the interactions of cyclodextrins and albumin subdomains with fullerenes (C60 and SWCNTs). Their predictions closely approached the results obtained with the NMR.

The computational methods for *in silico* prediction were also applied to nanosized metal oxides. Bakhtiyor, Leszczynska, and Leszczynski (2012) and Puzyn (2011) recently predicted *in silico* the cytotoxicity of 17 differently sized metal oxides. The authors reformulated an earlier published algorithm, based on quantum-chemical methods in combination with the nano-QSAR (quantitative structure–activity relationship). The adaptive Poisson–Boltzmann solver (APBS) software (Baker *et al.*, 2001), and the PROPKA, originally written to calculate the pK_a of proteins (Li, Robertson, and Jensen, 2005), successfully resolved the pK_a also for metal oxide NPs. The theoretical model developed

Table 10. Publicly accessible databases for nanotoxicity and toxicology. The Nano-EHS Database Analysis Tool is provided as a part of the *Virtual Journal of Nanotechnology Environment, Health and Safety* by the publisher Wiley Interscience.

Name	Authority	Public release	Description	URL
Nano Archive	ICPC NanoNet	2008	A database publications, with a section dedicated to the risk of nanotechnology	http://www.nanoarchive.org/
Iuclid 5.2	ECHA	2010	An inventory for classification and labeling of NMs is included	http://echa.europa.eu/
JRC NanoHub	IHCP at JCR	2009	Data are in accordance with the OECD and ISO guidelines	http://napira.jrc.ec.europa.eu/
NHECD	JRC	2009	The search includes descriptors implemented by the server. The output, summarized in a downloadable Excel sheet, includes bibliography on nanotoxicology	http://nhecd.jrc.ec.europa.eu/
RSMN DB ^a	OECD	2009	The search string retrieves research projects and can be extended to bibliography (ICON), and to documents issued at the NIOSH	http://webnet.oecd.org/ NanoMaterials/Pagelet/ Front/Default.aspx
NanoEHS DB Analysis Tool	ICON	2011	The tool offers a series of choice (time limits since 1962 and descriptors). The output provides the citation, the link to the text and the pay chart, or the histogram of time progressive distribution analysis. Download as .pdf (or Excel) file	http://icon.rice.edu/research.cfm

ECHA, European Chemical Agency; ICON, International Council of Nanotechnology; ICPC, International Co-operation Partner Countries; JCR, Joint Common Research Center, Ispra, Italy; Nano-EHS DB, Nanotechnology Environment, Health and Safety; NHECD, Nanoparticles Health and Environmental Commented Database; NIOSH, National Institute for Occupational Safety and Health; RSMN DB: Database on Research into the Safety of Manufactured Nanomaterials.

^aLaunched by the OECD on 1 April 2009.

by Burello and Worth (2011a) predicted the ability to induce oxidative stress for 64 of metal oxide NPs. Xia, Monteiro-Riviere, and Riviere (2010) proposed the biological surface adsorption index, an integrated parameter derived from five descriptors, namely acidic/basic properties of hydrogen bonds, polarity/polarizability, excess molar refraction, Coulomb forces, and lone-pair electrons. The authors presented the predictions for 12 NMs. The plot on a radar compass returned a profile unique for each of them, proving the accuracy of the method. In a further work, Xia *et al.* (2011) derived the index by principal component analysis for 16 NMs, whose clustering pattern was highly significant (Xia *et al.*, 2011). The advancements in nanotoxicology based on quantum chemistry computation have been summarized in recent reviews (Burello and Worth, 2011b, c; Saliner, Poater, and Worth, 2008).

A model was integrated into a software available since 2009 (multiple-path particle dosimetry model, MPPD v 2.11) for the prediction of the distribution and dosimetry of particle (from 10 to 20 000 nm) in

the airways of human and rat. The algorithm calculates the efficiency of deposition and sedimentation of mono and polydisperse aerosol in the different airways. The calculation includes the dynamics due to the impact of filtration through the nose and mouth, that of bifurcation, and the influence of the clearance. Adaptation of the software to provide prediction in children is also available. The output is both a plot and a graphical view, through a friendly-to-use interface (Anjilvel and Asgharian, 1995; National Institute for Public Health and the Environment, 2002).

Databases specialized in nanotoxicology studies and engines for automatic search represent an attempt to distribute instrument for a rapid insight into the field. Table 10 lists the web sites hosting these instruments. Taken as a whole, the advancement of knowledge in the field of computational nanotoxicology traces a very exciting and evolutive picture for future studies. Consequently, the expectancy for a rapid evolution of *in silico* predictability seems to be realistic.

9 CONCLUSION

The still rare, but dramatic, reports of nanotoxicity occurrences in humans urge for better prevention of accidental or professional exposure. Great efforts in this prospect were done in recent years, whereas the gap between knowledge and cognizable still exists. Standard materials became finally approved by control agencies and are commercially available. The standardization of biological models for studies *in vitro* and *in vivo* improved, and it is now possible to differentiate the risk hazard according to the modalities of exposure. The advancement in ecotoxicology applied to NMs and studies on developmental toxicity, in model organisms and mammals, as NMs are able to cross the placenta. Suitable models of differential exposure reproduced, *in vitro* and *in vivo*, the main route of administration or of access to the organism and provided appropriate description of bioavailability, distribution, and systemic or targeted toxicity hazard. The description of the interactions of several NMs with plasma proteins and the following biological reactions were described and even modeled, whereas computational methods for predictive nanotoxicology are still at their beginning.

Nevertheless, and in spite of the explosive growth of studies in the field of nanotoxicology, this science retains an overall aspect of yet fragmentary knowledge, dispersed upon poor standardized procedures and large number of manufactured materials. A great part of them shares the chemical nature and even the generic name, despite differences in size, shape, surface, and toxic residuals of production. As an example, the category “single- or multi-walled carbon nanotubes” does not reveal about fundamental properties such as chirality, shape of terminal parts, and functionalization. The development of robust knowledge in the nanotoxicology field mainly depends on the standardization of the phases of production, experimental exposure, and the availability of well-characterized and worldwide-distributed standard materials. Approved models and methods of study are of great importance. The work of regulatory agencies and scientist seems to be far from its completeness.

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Considerations for *In Vitro* Nanotoxicity Testing

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1 INTRODUCTION

Nanotechnology is defined by the National Nanotechnology Initiative as the understanding and control of matter at dimensions between approximately 1 and 100 nm, where unique phenomena enable novel applications (National Science and Technology Council Committee on Technology Subcommittee on Nanoscale Science, 2011). Nanoparticles (NPs) are loosely defined as having at least one dimension in the range ~1–100 nm and all dimensions measuring <1 µm, although there is considerable debate on this definition (Farokhzad and Langer, 2009; Anton, Benoit, and Saulnier, 2008; Christian *et al.*, 2008; Auffan *et al.*, 2009).

The United States Food and Drug Administration (USFDA) has not officially adopted a definition for nanotechnology, nanomedicine, or related terms. The agency, however, has issued a draft guidance aimed at articulating the principles that sponsors could use when determining whether a product contains nanomaterials. Specifically, the draft USFDA guidance lists considerations as follows—when deciding whether an USFDA-related product contains nanomaterials or otherwise involves the application of nanotechnology, the USFDA will ask the following information:

1. Whether an engineered nanomaterial or end product has at least one dimension in the nanoscale range (approximately 1–100 nm).

2. Whether an engineered nanosized material or end product exhibits properties or phenomena, including physical or chemical properties or biological effects that are attributable to its dimension(s), even if these dimensions fall outside the nanoscale range, up to 1 µm (USFDA, 2011).

NPs are being developed for a variety of therapeutic applications, including drug delivery, disease diagnostics, and imaging agents (Keene *et al.*, 2010; Chua *et al.*, 2011; Nitin, Javier, and Richards-Kortum, 2007). One of the advantages of using NPs for drug delivery is the potential for a single NP to be used simultaneously to improve the water solubility of a drug, target the drug to a particular tissue, co-deliver multiple drugs, and visualize the drug delivery using an imaging agent (Farokhzad and Langer, 2009). Money spent on the advancement of nanotechnology for the biomedical field continues to increase as the field matures (Datar and Cote, 2010; Tinkle, 2010).

While the biomedical field continues to expand applications of NPs for therapeutic uses, research differs on whether these materials are “safe” (Nel *et al.*, 2006; Service, 2005; Park *et al.*, 2009). One issue confounding safety assessment is that there exist contradictory observations of toxicity of these materials in the literature, even when size, composition, and testing methods are controlled.

It is expected that the toxicity of a given NP will depend on its physicochemical properties such as its chemical composition, size, shape, surface area, and terminal surface (Lewinski, Colvin, and Drezek, 2008; Ispas *et al.*, 2009; Petushkov *et al.*, 2009; Fadeel and Garcia-Bennett, 2010). However, before the results of any nanotoxicology studies can be compared, the assays used in these studies must be assessed for their ability to accurately predict the toxicity of NPs. This chapter discusses *in vitro* nanotoxicity testing, including assay setup, experimental conditions, and assay interference conditions.

2 CONSIDERATIONS BEFORE TESTING

2.1 External Factors

External (non-nano) factors were some of the most widespread causes of erroneous assay results as researchers first began applying *in vitro* assays to NP constructs. These external factors, such as solvent effects, biologically contaminated samples, and plating bias, are considered “non-nano” factors as these issues are not limited to NP systems but may occur for any entity applied to these *in vitro* assays.

One of the first sources of error with any testing method is user error when applying an NP construct to *in vitro* assays. A portion of this problem may be due to the fact that, initially, many of the laboratories using these assays were familiar either with the tested nanomaterials or with *in vitro* assays, but few laboratories originally had expertise in both. These differences in expertise lead to many mistakes in assessing NPs' toxicity. For instance, the use of solvent or stabilizing agents that prevent agglomeration and/or aggregation of NPs is important when conducting *in vitro* assays. Some of these solvents or stabilizing agents, however, may be toxic to the cells even if the NPs themselves are not toxic. For instance, dimethyl sulfoxide (DMSO) was found to stabilize copper oxide NPs during synthesis (Rout, Jammi, and Punniyamurthy, 2007). However, concentrations of as low as 1% DMSO decrease cell viability *in vitro* (Cao *et al.*, 2007). In addition, solid lipid NPs have been stabilized with surfactants such as Tween 20 and sodium dodecyl sulfate (SDS), which significantly increases the toxicity of these NPs to cells (Muller *et al.*, 1997;

Mehnert and Mader, 2001). Specialists in *in vitro* assays will often know what surfactants and/or solvents are toxic to the cells used with a particular *in vitro* assay, but they may not be familiar with the specific physicochemical properties that are unique to each NP and may report invalid *in vitro* results because of assay interference that is not obvious to the experimenter. For instance, NPs frequently have absorbance or fluorescent properties that would not be expected for a small molecule being tested with an *in vitro* toxicity assay. The NPs' absorbent or fluorescent wavelengths may overlap with the wavelength measured by the assay, invalidating the assay results (Kroll *et al.*, 2009). Often, the only way to measure if a particular NP interferes with a particular assay is by running a series of controls, which are discussed later in this chapter.

Before investigating any suspected (or unsuspected) NP assay interference, it is necessary to ensure the identity, strength, quality, and purity of the NP formulation itself (Jones *et al.*, 2008). The physicochemical characterization metrics recommended for NP formulations have been reviewed elsewhere and include establishing the chemical composition, size, size distribution, agglomeration status, shape, and surface chemistry of the NPs (Hall *et al.*, 2007; Warheit, 2008). Each of these features can directly or indirectly impact assay performance [e.g., agglomeration of gold NPs causes a shift in the absorbance spectrum of the NPs (Rivera *et al.*, 2010)]. Often, NPs are characterized in their pristine state (i.e., before particles have been exposed to biological conditions). However, more and more characterization recommendations are focusing on biological characterization, where data are measured under conditions mimicking the end point application (Lam *et al.*, 2006; Sayes and Warheit, 2009). Such characterization is increasingly useful when beginning to assess assay interference as characteristics such as surface opsonization and NP dissolution or agglomeration/aggregation, which may directly impact assay results.

Along with the physicochemical properties of the NPs, purity of the NP formulation to be tested may have a significant impact on the performance of *in vitro* assays. There have been several reports of the impurities in an NP formulation causing erroneous assay data. For example, unprocessed carbon nanotubes may contain up to 50% metal impurities (by weight) and can cause enhanced toxicity not directly associated with the NP itself

(Lam *et al.*, 2006). Stabilizers within the NP formulation may also cause interference with assays. For example, cetyltrimethylammonium bromide (CTAB), a capping agent used to stabilize gold NPs, has been shown to cause toxicity to the K562 cell line both alone and when stabilizing NPs. However, when the particles were repeatedly centrifuged to remove the CTAB, they did not appear toxic to the cell line (Nel *et al.*, 2006). The degradation of the NPs may also interfere with results. Quantum dots were found to be nontoxic while intact, but under certain conditions, a deterioration of the CdSe core resulted in acute toxicity (Derfus, Chan, and Bhatia, 2004).

Solvent effects are another issue that can significantly impact the outcome of *in vitro* assays. Certainly, the use of a solvent that is not compatible with living systems will cause enhanced mortality *in vitro*. Removal of these solvents and transfer into a biologically compatible solvent may introduce trace amounts of the toxic solvent or other impurities to the formulation. For example, side products formed during the suspension of fullerenes in tetrahydrofuran (THF) and subsequent transfer to water resulted in acute toxicity in A549 cells that was attributed to side products formed during the suspension process. The toxicity was not observed after several washing steps (Spohn *et al.*, 2009).

The concentration of the NPs dispersed within the solvent may also contribute to false results. Care should be taken to ensure that the solvent itself is not causing any observed cellular effects. For example, a dilute aqueous solution of NPs when used in a dose response manner could dilute the media to such an extent that the osmotic balance of the cells is disrupted, and changes in viability markers are observed due to this effect (Keene *et al.*, 2011). A simple control for such interference is to run a vehicle control at the same time as the NP formulation. Another control would be to remove the NPs, by filtration, centrifugation, or some other method, and examine the effect of the filtrate and retentate separately (Oostingh *et al.*, 2011).

Sterility and endotoxin contamination are two other major issues in NP toxicological assays. Viral, bacterial, yeast, mycoplasmal, and/or fungal contamination, especially at levels not readily visualized, may change performance of cell lines (Freshney, 2005). In addition, endotoxin contamination has been observed to be especially prevalent for NP formulations and has been shown to trigger

monocyte reactions irrespective of the NP platform (Pfaller *et al.*, 2010). Endotoxins are lipopolysaccharides (LPSs) found in the outer membrane of Gram-negative bacteria that may cause fever, decrease blood pressure, and activate inflammation pathways upon administration. Although an NP formulation may have gone through a sterilization procedure, it may still be contaminated with endotoxin, which can lead to erroneous assay results. Diagnosing NP endotoxin contamination has its own set of assay interference issues including protein binding and colorimetric interference (Dobrovolskaia *et al.*, 2010). Only after an NP solution has been deemed sterile and endotoxin free should it be applied to an *in vitro* test system.

Finally, the overall layout of an assay plate is a factor that can impact assay results. Plate edge effects, in particular for assays run longer than 24 h, may impact results by the edges of the plate having more evaporation than the internal wells, leading to nonuniform dosing of the chemicals and assay reagents, concentrated media ingredients, and so on (Caux *et al.*, 1992). Temperature gradients across the plates may also result in edge effects (Burt, Carter, and Kricka, 1979). Running statistical analysis on a single exposure point across an entire plate at the time points of interest will help determine the effects of any plating bias.

2.2 General NP Assay Interference

Commercially available cytotoxicity and other *in vitro* kits are often used as an initial screen to assess toxicity for many products, be it small molecule or NP. Advantages of these kits include the ability to test many compounds and concentrations simultaneously using high-throughput methods, the advantage for a “quick” toxicity response, and the cost and time savings associated with using cells and cell lines as opposed to animal models (Bakand *et al.*, 2005; Parvez, Venkataraman, and Mukherji, 2006; Collins, Gray, and Bucher, 2008). Applying these *in vitro* assays to NP constructs, however, is not as straightforward as applying them to small molecules. NPs have been noted to interact with all aspects of *in vitro* assay mechanisms, resulting in a wide array of false positives and false negatives (Casey *et al.*, 2007a,b; Laaksonen *et al.*, 2007; Monteiro-Riviere, Inman, and Zhang, 2009). As NPs frequently interfere with *in vitro* assays,

the toxicity of NPs in the literature often shows conflicting results.

As the problems with applying *in vitro* assays to NP continue to surface, several laboratories have begun preparing lists of NPs that interfere with common *in vitro* assays, controls to probe for such interference, and possible solutions to these problems. For instance, a study compared six assays for measuring cell viability with five different NP constructs. The study found that each of the NPs caused some interference with at least one of the assays, usually because of absorbance of the NPs overlapping with the absorbance of the signal measured in the assay (Monteiro-Riviere, Inman, and Zhang, 2009). In addition, a recent review listed seven common assays used to measure *in vitro* toxicity and the types of interference that an NP may cause. The methods of interference varied from absorption of the assay components by the NP to fluorescent quenching caused by the nanomaterial. Each of these methods of interference led to false positive (perceived increase in toxicity) or false negative (perceived decrease in toxicity) results. Controls to be run concurrently with the assay were suggested to assist in determining whether a particular NP was interfering with the given assay (Kroll *et al.*, 2009). These controls are further expanded in another paper, which lists several controls that should be used with all *in vitro* assays involving NPs. These controls include a reagent known to produce a positive effect and a reagent known to produce a negative effect for the assay (Stone, Johnston, and Schins, 2009). In addition, several groups have begun to look at the effects of NP interference on particular class of *in vitro* toxicity assays. For instance, two papers were published on the issues of applying NPs to genotoxic assays (Donaldson, Poland, and Schins, 2010; Pfaller *et al.*, 2010). Finally, the Nanotechnology Characterization Laboratory (NCL) has produced a series of papers on importance of controls required to avoid false negative and false positive results when using NP in immunotoxic and sterility assays (Hall *et al.*, 2007; Dobrovolskaia *et al.*, 2008a, b, 2010; Dobrovolskaia, Germolec, and Weaver, 2009).

While these articles begin to identify and study the problems associated with assay interference when applying *in vitro* assays to NPs, this chapter attempts to highlight the many types of NP interference seen with *in vitro* assays as well as to list the appropriate controls depending on the type of

interference found. Table 1 provides a list of NP interference observed with *in vitro* assays. The table lists the assays in which the particular type of interference was or could be seen, the type of NP responsible for the interference, and the effect of the interference. In all cases, *positive interference* is listed as a false increase in reported toxicity, whereas *negative interference* is listed as a false decrease in reported toxicity. Finally, the table includes a list of suggested methods to control and compensate for certain types of interference problems.

3 SPECIFIC FACTORS IN ASSAY INTERFERENCE

Assay interference may occur through a variety of mechanisms. Here, we detail some of the more prevalent causes of assay interference.

3.1 Physical Interference

Physical interference includes NPs settling out of solution and causing inconsistent dosing and/or analysis as well as turbid solutions resulting in a high measurement baseline (Hall *et al.*, 2007; Dobrovolskaia, Germolec, and Weaver, 2009; Murthy and Pathak, 2009). NP agglomeration/aggregation into large superstructures may also cause physical interference. Specific examples of these physical factors have been noted in the literature. For example, NP self-association in physiological media has been shown to prevent accurate cell counts in trypan blue viability assays (Monteiro-Riviere, Inman, and Zhang, 2009). In some assays, aggregates/agglomerates may be miscounted as other features, such as aggregated platelets in a platelet aggregation assay (Hall *et al.*, 2007). NPs known to form aggregates and agglomerates in biological media such as saline include gold NPs and single-walled carbon nanotubes (SWCNTs) (Dobrovolskaia *et al.*, 2008a; Keene and Tyner, 2011; Niyogi *et al.*, 2007). In other cases, the NPs may physically block the test from being read/analyzed, such as in the case of the micronucleus assay and carbon nanotubes and TiO₂ NPs (Falck *et al.*, 2009; Lindberg *et al.*, 2008). Aggregation/agglomeration and settling effects may also affect NP dosimetry, influencing *in vitro* results (Teeguarden *et al.*, 2007). Finally, NP

Table 1. Common assay interference.

Assay type	Assay	Examples of NPs that cause interference	Mechanism of interference	Effect	Control	References
Immunotoxic	Hemolysis	Gold NP, fullerene, and nanoemulsions	Absorbance of NP increases overall absorbance	Positive (increased signal appears as increased hemolysis)	Measure absorbance of NPs without cells	Murthy and Pathak (2009), Dobrovolskaia <i>et al.</i> (2008b), Hall <i>et al.</i> (2007)
Immunotoxic	Hemolysis	Iron oxide NPs	NPs oxidize assay reagents	Positive (increased signal appears as increased hemolysis)	Measure absorbance before and after spiking with NPs	Dobrovolskaia <i>et al.</i> (2008b)
Immunotoxic	Hemolysis	Gold and polystyrene NPs	NPs absorb hemoglobin	Negative (decreased hemoglobin appears as decreased hemolysis)	Measure absorbance before and after spiking with NPs	Dobrovolskaia <i>et al.</i> (2008a, b), Hall <i>et al.</i> (2007)
Immunotoxic	Hemolysis	Dendrimers	NPs cause blood clots that prevent hemolysis	Negative (NPs prevent hemolysis from occurring by coagulating blood)	Check for platelet aggregation in addition to measuring absorbance	Dobrovolskaia <i>et al.</i> (2008b)
Immunotoxic	Phagocytosis	Quantum dots	Luminescent NPs increase luminescence of assay	Positive (increased luminescence shows increased phagocytosis)	Measure luminescence before and after spiking with NPs	Murthy and Pathak (2009)
Immunotoxic	Phagocytosis	Gold NPs and liposomes	NPs quench luminescence of assay	Negative (quenched luminescence shows decreased phagocytosis)	Measure luminescence before and after spiking with NPs	Dobrovolskaia, Germolec, and Weaver (2009), Hall <i>et al.</i> (2007)
Immunotoxic	Platelet aggregation	Gold NPs and fullerenes	NP aggregates counted as aggregated platelets	Positive (number of platelet aggregates counted increased)	Look for NP aggregates	Hall <i>et al.</i> (2007)
Immunotoxic	Platelet aggregation	Gold NPs and fullerenes	NP aggregates with platelets so that platelets counted as cells	Negative (number of platelet aggregates appears to be decreased)	Look for NP aggregates	Hall <i>et al.</i> (2007)
Immunotoxic/ inflammation	Cytokine ELISA	Dendrimers	NPs prevent protein–protein binding	Negative (cytokines not quantified so inflammation underestimated)	Measure cytokine in NP sample spiked with cytokine protein	Dobrovolskaia <i>et al.</i> (2008a)

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Table 1. (Continued)

Assay type	Assay	Examples of NPs that cause interference	Mechanism of interference	Effect	Control	References
Immunotoxic/inflammation	Cytokine ELISA	CB NPs, metal oxide NPs, SWCNT, iron oxide NPs, copper NPs, SiO ₂ , Al ₂ O ₃ , CeO ₂ , NiO, TiO ₂ , and diesel NPs	NPs absorb cytokine protein	Negative (NPs absorb cytokine protein, so that it is not detected by ELISA)	Measure cytokine in NP sample spiked with cytokine protein	Monteiro-Riviere <i>et al.</i> (2006), Kroll <i>et al.</i> (2009, 2012), Guo <i>et al.</i> (2008), Brown <i>et al.</i> (2010), Kocbach <i>et al.</i> (2008), Val <i>et al.</i> (2009), Veranth <i>et al.</i> (2007), Davoren <i>et al.</i> (2007), Seagrave <i>et al.</i> (2004)
Immunotoxic	PCR of IL-1 β or caspase-3 induction in gut cells	Gold and iron oxide NPs	NP solvent causes gene upregulation	Positive (genes appear upregulated when they are not)	Measure upregulation caused by NP synthesis solution without NPs	Pfaller <i>et al.</i> (2010)
Endotoxin	Turbidity LAL	Liposomes, nanoemulsions, and polymers	NPs increase turbidity	Positive (NPs increase turbidity so more LDL appears present than actually is)	Measure turbidity of NPs without LAL	Dobrovolskaia, Germolec, and Weaver (2009), Murthy <i>et al.</i> (2009)
Endotoxin	Turbidity LAL	DOTAP-nanoliposomes and gold NPs	NPs bind LPS	Negative (NPs prevent LPS-LAL interaction)	Measure turbidity assay with NPs spiked with LPS	Dobrovolskaia, Germolec, and Weaver (2009)
Endotoxin	Turbidity LAL	Dendrimers and iron oxide NPs	NPs catalyze enzymatic reactions in assay	Positive (NPs catalyze reaction independent of LAL)	Measure reaction without LAL	Dobrovolskaia, Germolec, and Weaver (2009), Dobrovolskaia <i>et al.</i> (2008a)
Endotoxin	Turbidity LAL	Gold NP	NP interacts with LAL enzyme	Negative (NP inactivates enzyme)	Measure turbidity assay with NP spiked with LPS	Dobrovolskaia, Germolec, and Weaver (2009)
Endotoxin	Gel clot LAL	Gold NP	NP interacts with LAL enzyme	Negative (NP inactivates enzyme)	Measure gel clot assay with NP spiked with LPS	Dobrovolskaia, Germolec, and Weaver (2009)

Table 1. (Continued)

Assay type	Assay	Examples of NPs that cause interference	Mechanism of interference	Effect	Control	References
Endotoxin	Gel clot LAL	Dendrimers and iron oxide NP	NP catalyzes enzymatic reactions in assay	Positive (NP catalyzes reaction independent of LAL)	Measure reaction without LAL	Dobrovolskaia, Germolec, and Weaver (2009), Dobrovolskaia <i>et al.</i> (2008a)
Endotoxin	Gel clot LAL	DOTAP-nanoliposomes and gold NP	NP binds LPS	Negative (NP prevents LPS–LAL interaction)	Measure gel clot assay with NP spiked with LPS	Dobrovolskaia, Germolec, and Weaver (2009)
Endotoxin	Chromogenic LAL	DOTAP-nanoliposomes and gold NP	NP binds LPS	Negative (NP prevents LPS–LAL interaction)	Measure absorbance assay with NP spiked with LPS	Dobrovolskaia, Germolec, and Weaver (2009)
Endotoxin	Chromogenic LAL	Gold NP	NP interacts with LAL enzyme	Negative (NP inactivates enzyme)	Measure absorbance assay with NP spiked with LPS	Dobrovolskaia, Germolec, and Weaver (2009)
Endotoxin	Chromogenic LAL	Gold NP, iron oxide NP, quantum dots, fullerenes, and liposomes	NP that absorbance at 405 nm increase absorbance	Positive (NP increases absorbance so more LDL appears present than actually is)	Measure absorbance of NP without LAL	Dobrovolskaia, Germolec, and Weaver (2009), Dobrovolskaia <i>et al.</i> (2008a), Oostingh <i>et al.</i> (2011)
Endotoxin	Chromogenic LAL	Dendrimers and iron oxide NP	NP catalyzes enzymatic reactions in assay	Positive (NP catalyzes reaction independent of LAL)	Measure reaction without LAL	Dobrovolskaia, Germolec, and Weaver (2009), Dobrovolskaia <i>et al.</i> (2008a)
Genotoxic	Cytokinesis-block micronucleus assay (CBMN) in monocytes	Gold NP and iron oxide NP	Endotoxin contaminant causes activation of monocyte reaction	Positive (LPS, not NP creates CBMN)	Measure upregulation caused by NP synthesis solution without NP	Pfaller <i>et al.</i> (2010)
Genotoxic	Comet assay	Gold NP and iron oxide NP	NP aggregates appear in tail of comet	Positive (DNA appears damaged when it is not)	Wash cells to remove as many aggregates as possible	Pfaller <i>et al.</i> (2010), Stone, Johnston, and Schins (2009)
Genotoxic	Comet assay	TiO ₂ NP and copper oxide NP	NPs autofluoresce to show a brighter comet head	Negative (NP is being detected rather than undamaged DNA)	Review after DNA stain has faded	Karlsson (2010)
Genotoxic	Comet assay	Germanium NP	NP interacts with DNA during the electrophoresis portion of the assay causing breaks	Positive (DNA appears damaged when it is not)	Confirm damage with secondary technique	Lin <i>et al.</i> (2009)

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Table 1. (Continued)

Assay type	Assay	Examples of NPs that cause interference	Mechanism of interference	Effect	Control	References
Genotoxic	Comet assay	TiO ₂ NP	NP quench DNA stain ethidium bromide	Neither (NP prevents proper cell analysis of entire assay)	Test ability of NP to quench DNA stain before running assay	Stone <i>et al.</i> (2009)
Genotoxic	Comet assay	TiO ₂ NP	NP causes DNA breakage when gels are processed in light because of the photoactivity of the NP	Positive (DNA appears damaged when it is not)	Process samples in dark	Gerloff <i>et al.</i> (2009)
Genotoxic	Comet assay	No specific example provided	NP interacts with properties of agarose gel	Undetermined as yet	None provided	Handy <i>et al.</i> (2012)
Genotoxic	Ames test	Multiple NP	Prokaryotic cell membrane prevents NP entry	Negative (NP does not cause DNA damage because they do not have access to prokaryote DNA)	Measure NP uptake in prokaryote system	Park <i>et al.</i> (2009)
Genotoxic	Micronucleus test	Multiple NP	Standard treatment with cytochalasin B causes NP to not be uptaken by cells	Negative (NP does not cause damage because they are prevented from entering the cell)	Modify assay to treat cells with NP before treatment with cytochalasin B	Magdolenova <i>et al.</i> (2012)
Genotoxic	Micronucleus test	Carbon nanotubes and graphite nanofibers; TiO ₂ NP	Carbon nanotubes on slides disturbed analysis and rendered test useless at doses higher than 20 µg cm ⁻²	Test is unable to be performed at high doses; negative (micronuclei are not able to be counted)	None provided	Lindberg <i>et al.</i> (2008), Falck <i>et al.</i> (2009)
Genotoxic	Comet or micronucleus test	Long non-biopersistent fibrous NP	NP causes genotoxicity due to short assay time but would be broken down by macrophages	Positive (NP only causes genotoxicity as cells do not have the mechanisms to digest the NP)	Measure biopersistence of NP <i>in vivo</i>	Donaldson, Poland, and Schins (2010)
Cell viability	MTT	Gold NP with CTAB	NP contaminants toxic but NP is not	Positive (CTAB increases cytotoxicity)	Measure solvent control from NP synthesis without NP	Fadeel and Garcia-Bennett (2010)

Table 1. (Continued)

Assay type	Assay	Examples of NPs that cause interference	Mechanism of interference	Effect	Control	References
Cell viability	MTT	Gold NP, CB, SWCNT, fullerene, sodium titanate NP, quantum dots, silver NP, silver oxide NP, and iron oxide NP	NP increases absorbance	Negative (more cells appear viable than actually are due to NP absorbance)	Measure absorbance of NP in media without cells	Malugin and Ghandehari (2010), Monteiro-Riviere, Inman, and Zhang (2009), Kroll <i>et al.</i> (2009, 2011), Stone, Johnston, and Schins (2009), Clift <i>et al.</i> (2008), Hall <i>et al.</i> (2007), Pujalte <i>et al.</i> (2011), Doak <i>et al.</i> (2009)
Cell viability	MTT	Silicon NP, SWCNT, fullerenes, and TiO ₂	NP reduces MTT	Negative (NP reduces MTT and appears to increase viability by increasing absorbance)	Measure absorbance of MTT and NP without cells	Laaksonen <i>et al.</i> (2007), Stone, Johnston, and Schins (2009), Belyanskaya <i>et al.</i> (2007), Low <i>et al.</i> (2006), Casey <i>et al.</i> (2007), Berg <i>et al.</i> (2010), Hall <i>et al.</i> (2007), Holder <i>et al.</i> (2012)
Cell viability	MTT	Zinc NP	NP prevents MTT reduction	Positive (decreased absorbance when MTT is not reduced implies cell death)	Spike assay with NP known to reduce MTT	Kroll <i>et al.</i> (2009)
Cell viability	MTT	Carbon black NP and SWCNT	NP absorbs MTT product	Positive (NP absorbs formazan decreasing absorbance so cells appear dead)	Measure absorbance formazan in media without cells	Monteiro-Riviere <i>et al.</i> (2006), Stone, Johnston, and Schins (2009), Worle-Knirsch, Pulskamp, and Krug (2006)
Cell viability	MTT	Fullerenes	Contaminants from NP processing were toxic, as opposed to the NPs	Positive (contaminants caused toxicity)	Additional washing of particles eliminated toxicity	Spohn <i>et al.</i> (2009)
Cell viability	MTT	Anti-oxidant NP	NP that interferes with drug efflux pumps prevents MTT from entering cell	Positive (decreased absorbance when MTT is not reduced implies cell death)	Measure NP uptakes in cellular system	Murthy and Pathak (2009)

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Table 1. (Continued)

Assay type	Assay	Examples of NPs that cause interference	Mechanism of interference	Effect	Control	References
Cell viability	MTT	Mesoporous SiO ₂ NP	Enhances MTT formazan exocytosis	Positive (increased absorbance indicates increased toxicity)	None provided	Fisichella <i>et al.</i> (2009)
Cell viability	MTT	Super-paramagnetic iron oxide NP	NP adsorbs Cl ⁻ ions, causing toxicity	Positive (cells have increased cell death due to depleted media)	Introduce NPs to cell media for 24 h and replace into fresh media before assay	Mahmoudi <i>et al.</i> (2010)
Cell viability	MTT	ZnO, SiO ₂ , carbon black, CeO ₂ , AlOOH, Ti-Zr, Ti-Al-Zr, BaSO ₄ , and SrCO ₃	Optical interference at high concentrations	Reduces MTT signal	Wash cell monolayers	Kroll <i>et al.</i> (2012)
Cell viability	XTT	Silicon NP	NP reduces XTT	Negative (increased absorbance implies increased cell viability)	Measure absorbance of MTT and NP without cells	Low <i>et al.</i> (2006)
Cell viability	MTS	Aluminum NP	NP aggregates and increases absorbance	Negative (increased absorbance implies increased cell viability)	Measure absorbance of NP in media without cells	Braydich-Stolle <i>et al.</i> (2005), Oostingh <i>et al.</i> (2011)
Cell viability	MTS	SWCNT	NPs deplete media of nutrients but are not themselves toxic	Positive (cells have increased cell death due to depleted media)	Supplement media with additional amino acids/ vitamins/ minerals and test viability; preincubate the NPs with media before testing	Guo <i>et al.</i> (2008), Horie <i>et al.</i> (2012)
Cell viability	Cell Titer Blue	Cobalt oxide NP	NP increases fluorescence	Negative (increased absorbance implies increased cell viability)	Measure absorbance of product with and without NP	Oostingh <i>et al.</i> (2011)

Table 1. (Continued)

Assay type	Assay	Examples of NPs that cause interference	Mechanism of interference	Effect	Control	References
Cell viability	WST-1	Iron oxide NP	NP increases absorbance	Negative (increased absorbance implies increased cell viability)	Measure absorbance of NP in media without cells	Ying and Hwang (2010)
Cell viability	LDH membrane leakage	Gold NP, SiO ₂ NP; iron oxide NP, and cesium oxide NP	NP increased absorbance	Positive (absorbance increased signifying more dead cells)	Measure absorbance of LDH in media without cells	Keene <i>et al.</i> (2011), Wahl <i>et al.</i> (2008), Kroll <i>et al.</i> (2011), Oostingh <i>et al.</i> (2011)
Cell viability	LDH membrane leakage	Carbon black NP, SWCNT, fullerene, and quantum dots	NP quenched fluorescent signal	Negative (fluorescence decrease implies more cells are viable)	Measure fluorescent signal of NP and media without cells	Monteiro-Riviere, Inman, and Zhang (2009)
Cell viability	LDH membrane leakage	Quantum dots	NP breakdown and components toxic	Positive (metal ions lead to increased toxicity)	None provided	Clift <i>et al.</i> (2008)
Cell viability	LDH membrane leakage	Copper NP, quantum dots, and TiO ₂ NP	NP absorbs LDH	Negative (decreased absorbance from LDH implies increased viability)	Measure absorbance of LDH spiked with NP without cells; lyse cells with LDH and measure absorbance before and after addition of LDH	Kroll <i>et al.</i> (2009), Stone, Johnston, and Schins (2009), Clift <i>et al.</i> (2008), Suska <i>et al.</i> (2005), Holder <i>et al.</i> (2012), Han <i>et al.</i> (2011)
Cell viability	LDH membrane leakage	ZnO and Cu NPs	Inhibition/inactivation of LDH activity	Negative (decreased absorbance from LDH implies increased viability)	None provided	Kroll <i>et al.</i> (2012), Han <i>et al.</i> (2012)
Cell viability	LDH membrane leakage	ZnO, SiO ₂ , carbon black, CeO ₂ , AlOOH, Ti-Zr, Ti-Al-Zr, BaSO ₄ , and SrCO ₃	Optical interference at high NP concentrations	Reduces LDH signal (substance appears less toxic)	Run positive control spiked with NP after initial plate reading	Kroll <i>et al.</i> (2012)

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Table 1. (Continued)

Assay type	Assay	Examples of NPs that cause interference	Mechanism of interference	Effect	Control	References
Cell viability	LDH membrane leakage	Silver NPs	LDH inactivation due to the presence of carbon matrix used during NP synthesis	Reduces LDH signal (substance appears less toxic)	None provided	Han <i>et al.</i> (2012)
Cell viability	Adenylate kinase	SWCNT	Absorption of adenylate kinase onto NP	Reduces AK signal (substance appears less toxic)	None provided	Davoren <i>et al.</i> (2007)
Cell viability	Propidium iodide	Gold NP	NP tracked dye into healthy cells	Positive (absorbance increased signifying more dead cells)	Visual observation with light microscope	Keene <i>et al.</i> (2011), Kroll <i>et al.</i> (2009)
Cell viability	Propidium iodide	Quantum dots and polystyrene NP	Fluorescent NP increases fluorescent signal	Positive (increased fluorescence indicates decreased viability)	Measure fluorescent signal of NP with dye without cells	Stone, Johnston, and Schins (2009)
Cell viability	Calcein AM	Carbon black NP and SWCNT	NP aggregate and block fluorescent signal	Positive (fluorescence decrease shows less viable cells)	Look for NP aggregates under microscope; measure fluorescent signal of NP and media without cells	Monteiro-Riviere, Inman, and Zhang (2009)
Cell viability	Live/Dead® (calcein AM, ethidium homodimer -1)	Carbon black and iron oxide NP	NP increases fluorescent signal	Negative (increased calcein AM fluorescence shows increased viability)	Measure fluorescence of calcein AM with NP in media without cells	Berg <i>et al.</i> (2010)
Cell viability	Live/Dead (calcein AM, ethidium homodimer -1)	Carbon black and SWCNT	NP aggregate and block fluorescent signal	Positive (fluorescence decrease shows less viable cells)	Look for NP aggregates under microscope; measure fluorescent signal of NP and media without cells	Monteiro-Riviere, Inman, and Zhang (2009)
Cell viability	Fluorescein diacetate	Iron oxide NP	Fluorescent NP increases fluorescent signal	Negative (increased fluorescence indicates increased viability)	Measure fluorescent signal of NP with dye without cells	Ying and Hwang (2010)

Table 1. (Continued)

Assay type	Assay	Examples of NPs that cause interference	Mechanism of interference	Effect	Control	References
Cell viability	ATP	SiO ₂ NP	NP quench luminescent signal	Positive (decreased luminescence indicates decreased viability)	Measure ATP luminescence before and after adding NP	Wahl <i>et al.</i> (2008)
Cell viability	Neutral red	SWCNT	NP quench dye	Positive (NP absorption and quenching leads to decreased absorbance/fluorescent and viability)	Measure absorbance/fluorescence of NR with NP in media without cells	Kroll <i>et al.</i> (2009), Casey <i>et al.</i> (2007a,b)
Cell viability	Neutral red	Carbon black, SWCNT, fullerene, silicon NP, and TiO ₂	NP absorbs neutral red (NR) dye	Negative (NP absorbs NR dye and increases absorbance to increase cells that appear alive)	Measure absorbance of NR with NP in media without cells	Monteiro-Riviere <i>et al.</i> (2006), Monteiro-Riviere, Inman, and Zhang (2009), Low <i>et al.</i> (2006), Casey <i>et al.</i> (2007a,b), Pujalte <i>et al.</i> (2011), L'Azou <i>et al.</i> (2008), Davoren <i>et al.</i> (2007)
Cell viability	Alamar Blue	Carbon black and SWCNT	NP quenched fluorescent signal	Positive (fluorescence decrease shows less viable cells)	Measure fluorescent signal of NP and media without cells	Monteiro-Riviere, Inman, and Zhang (2009), Casey <i>et al.</i> (2007a,b)
Cell viability	Alamar Blue	SWCNT	NPs deplete nutrients from media but are not themselves toxic	Positive (cells have decreased viability in depleted media)	Supplement media with additional amino acids/vitamins/minerals and test viability	Casey <i>et al.</i> (2008)
Cell viability	Alamar Blue	Silicon NP	NP reduces dye	Negative (increased fluorescence indicates increased viability)	Measure fluorescent signal of NP and media without cells	Low <i>et al.</i> (2006)

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Table 1. (Continued)

Assay type	Assay	Examples of NPs that cause interference	Mechanism of interference	Effect	Control	References
Cell viability/apoptosis	Caspase-3	Zinc NP	NP inhibits caspase-3 enzyme	Negative (inhibition of caspase-3 leads to decreased fluorescence and appearance of less apoptosis)	Measure assay with NP and spiked caspase-3	Kroll <i>et al.</i> (2009)
Cell viability/apoptosis	Annexin V	Chitosan NP	NP depletes calcium to prevent Annexin V binding	Negative (depletion of Annexin V binding signifies less apoptosis)	Measure calcium in media before and after adding NP	Kroll <i>et al.</i> (2009)
Cell viability/cell proliferation	Clonogenic assay	SWCNT	NPs deplete nutrients from media but are not themselves toxic	Positive (cells produce smaller colonies due to depleted media)	Supplement media with additional amino acids/vitamins/minerals and test viability	Casey <i>et al.</i> (2008)
Oxidative stress	Electroparamagnetic resonance (EPR)	TiO ₂ NP	NP chemically or physically interferes with spin trapping	Positive (NP decreases EPR signal)	Spike ROS donor system with NP	Stone, Johnston, and Schins (2009)
Oxidative stress	Ferric reducing ability of plasma	Silver oxide NP, fullerene, and TiO ₂ NP	NP increases absorbance	Positive (increased absorbance shows increased ROS)	Spike some NP solutions with antioxidant and determine if absorbance decreases	Rogers <i>et al.</i> (2008)
Oxidative stress	DCF	TiO ₂ NP, SWCNT, fullerene, SiO ₂ NP, carbon black NP, iron oxide NP, and gold NP	NP increases DCF fluorescence	Positive (increased fluorescence shows increased ROS)	Measure DCF fluorescence with NP without cells	Kroll <i>et al.</i> (2009, 2011), Stone, Johnston, and Schins (2009), Berg <i>et al.</i> (2010), Pfaller <i>et al.</i> (2010)
Oxidative stress	DCF	Fullerenes	Contaminants from NP processing were toxic, as opposed to the NPs	Positive (contaminants caused toxicity)	Additional washing of particles eliminated toxicity	Spohn <i>et al.</i> (2009)

Table 1. (Continued)

Assay type	Assay	Examples of NPs that cause interference	Mechanism of interference	Effect	Control	References
Oxidative stress	DCF	ZnO, SiO ₂ , carbon black NP, CeO ₂ , AlOOH, Ti-Zr, Ti-Al-Zr, BaSO ₄ , and SrCO ₃	Optical interference at high concentrations of NPs	Reduced DCF signal	Wash cell monolayers	Kroll <i>et al.</i> (2012)
Oxidative stress	DCF	Carbon black NP	Catalyzed reaction with no cells present	False increase of DCF signal	None provided	Kroll <i>et al.</i> (2012)
Oxidative stress	DCF	Ultrasmall superparamagnetic iron oxide NP	Both quenching and fluorescent increase have been reported, depending on oxidation state	False signal (positive and negative) of DCF	Measure fluorescence before and after addition of NPs	Doak <i>et al.</i> (2009)
Oxidative stress	DCF	Carbon NP	NP quench DCF fluorescence	Negative (decreased fluorescence shows decreased ROS)	Measure DCF fluorescence before and after addition of NP	Kroll <i>et al.</i> (2009)
Bacterial cell viability	Flash assay	Copper oxide NP and ZnO NP	Settling of NP decreases luminescence	Positive (decreased luminescence indicates decreased viability)	Compare microplate and cuvette assay as cuvette assay uses shaking that prevents NP settling	Mortimer <i>et al.</i> (2008)
Bacterial cell viability	Flash assay	Copper oxide NP and ZnO NP	Fluorescent/ luminescent NP increases luminescent signal	Negative (increased luminescence indicates increased viability)	Measure luminescence of NP without luminescent dye	Mortimer <i>et al.</i> (2008)
pH	Phenol red	Carbon nanotubes	Phenol red adsorbed to the NPs, reducing the color in the media	Media appears more acidic than it actually is	Measure the pH through alternate means	Casey <i>et al.</i> (2007a,b)
pH	Electrode-based	Many (none specified)	NPs absorbing to the electrodes, interference with the solutions or gels inside the probes, creation of spurious voltages	Both positive and negative	Use a mixed calibration approach	Handy <i>et al.</i> (2012)

The table lists assays for which NP interference has been observed. In all cases, positive interference is listed as a false increase in reported toxicity, whereas negative interference is listed as a false decrease in reported toxicity.

aggregates/agglomerates may localize in the tail of the comet assay, affecting the quantification of this assay (Stone, Johnston, and Schins, 2009); however, a recent study by Magdolenova *et al.* (2012), did not see such an effect with silica, titanium dioxide, poly(lactic-co-glycolic acid), magnetite, or iron oxide NPs. Other studies have found that NPs may localize in the head of the comet, making the comet head appear brighter due to NP autofluorescence (Karlsson, 2010). In general, a no cell control, with the NPs exposed to all other conditions, will often provide adequate control from this type of NP interference. In the case of turbidity effects, subtracting out baseline absorbance may sometimes be an appropriate fix.

3.2 Reporting Interference

Reporting mechanisms refer to the way that the assay end point is determined. Many assays use changes in absorbance, fluorescence, or luminescence in order to quantify the results of the toxicity measurement. However, NPs often have their own absorbance, fluorescence, and luminescence properties. The absorbance spectrum of many NPs including gold NPs, iron oxide NPs, quantum dots, fullerenes, and liposomes were found to overlap with 405 nm, the absorbance at which the chromogenic limulus amoebocyte lysate (LAL) assay is measured (Dobrovolskaia *et al.*, 2008a; Dobrovolskaia, Germolec, and Weaver, 2009; Oostingh *et al.*, 2011). Similarly, many NPs, particularly metal NPs, were found to increase the absorbance of the lactose dehydrogenase (LDH) assay, measured at 490 nm, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, measured at 540–590 nm, or the 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate (WST-1) assay, measured at 420–480 nm. These NPs include gold NPs, quantum dots, silver NPs, silver oxide NPs, iron oxide NPs, silica NPs, cesium oxide NPs, and SWCNTs (Monteiro-Riviere, Inman, and Zhang, 2009; Kroll *et al.*, 2009, 2011; Hall *et al.*, 2007; Keene *et al.*, 2011; Oostingh *et al.*, 2011; Malugin and Ghandehari, 2010; Clift *et al.*, 2008; Wahl *et al.*, 2008; Ying and Hwang, 2010). The increased absorbance leads to a positive interference in the LDH assay as more cells appear to have released LDH and to a negative interference in the MTT and WST-1 assays as more cells appear

to be viable than actually are. Other assays with absorbance readouts that may experience colorimetric interference include the hemolysis assay and the ferric reducing ability of plasma assay (Dobrovolskaia *et al.*, 2008b; Murthy *et al.*, 2009; Rogers *et al.*, 2008). In addition, it is important to note that in some cases, while the NP alone may not interfere with the absorbance of an assay, NP aggregates/agglomerates that form in biological solutions, such as cell culture media or buffered saline solutions, will have their own absorbance spectrum that could overlap with the wavelength measured in the assay. This phenomenon was seen with gold NPs and fullerenes in the hemolysis assay (Dobrovolskaia *et al.*, 2008b) and with aluminum NPs in the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay (Braydich-Stolle *et al.*, 2005).

NPs may also have fluorescent and luminescent properties in addition to absorbance properties. Even if the NP does not appear to have fluorescent properties, it may increase/change the fluorescent signal of a given assay either through fluorescent resonance energy transfer (FRET) or through some other mechanism. For instance, many NPs, including titanium dioxide NPs, fullerenes, silica NPs, carbon black NPs, iron oxide NPs, gold NPs, and SWCNTs, were found to increase the fluorescent output in the dichlorofluorescein (DCF) assay (Kroll *et al.*, 2009, 2011; Stone, Johnston, and Schins, 2009; Pfaller *et al.*, 2010; Berg *et al.*, 2010). This is an example of positive interference where the cells appear to be producing more ROS than they actually are. Other affected assays include the Cell Titer Blue[®] assay and the Live/Dead[®] Cell Viability assay (Oostingh *et al.*, 2011; Berg *et al.*, 2010). In both cases, the compounds would appear less toxic than they actually are due to the increased fluorescence. Luminescent assays also may have interference. Copper oxide NPs were found to interfere with the luminescent signal of the Flash Assay[®], which measures toxicity of the compound to bacteria (Mortimer *et al.*, 2008). Finally, many NPs that are being developed for drug delivery also use fluorescent trackers so that the NP drug conjugate may be imaged *in vitro* and *in vivo*. The new properties of the NPs after the conjugation process also need to be accounted for when completing *in vitro* toxicity assays.

NPs or NP aggregates/agglomerates may also block or quench fluorescent and luminescent signals, preventing an accurate reading for a given assay. Carbon NPs, such as SWCNT, were found to quench the fluorescent signal of calcein AM, resazurin (Alamar Blue[®]), propidium iodide, DCF, and neutral red (NR) dye assays (Casey *et al.*, 2007a,b; Monteiro-Riviere, Inman, and Zhang, 2009; Kroll *et al.*, 2009; Stone, Johnston, and Schins, 2009). In the cases of the calcein AM, Alamar Blue, and NR assays, this would lead to positive interference as the NPs would appear more toxic because of the decrease in signal. However, in the cases of propidium iodide and DCF assays, the compounds would appear less toxic. In another example, the signal in a luminescent version of an adenosine triphosphate (ATP) assay was found to be quenched by silica NPs, leading to a reported decrease in viability (Wahl *et al.*, 2008).

3.3 Chemical Reactions

Chemical reactions arising from NPs may also cause assay interference. NPs can catalyze assay reactions or directly react with assay components, producing false positive or false negative results. Owing to the varying chemical characteristics of different NP classes, there are several methods in which NPs can chemically interfere with *in vitro* assays. One of the most commonly reported mechanisms of interference is oxidation or reduction of components of the assay by NPs. For example, silicon NPs were found to reduce MTT, 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide (XTT), and Alamar Blue dyes in the absence of cells (Laaksonen *et al.*, 2007; Low *et al.*, 2006). In addition, SWCNTs were also found to reduce MTT in the absence of cells (Belyanskaya *et al.*, 2007). Each of these NPs caused a false negative response as the number of living cells appeared to be increased because of the excess amount of measured product. NPs may also cause positive interference by preventing the reduction of MTT. This mechanism of interference was reported for NPs containing metal ions (Kroll *et al.*, 2009). Finally, iron oxide NPs have also been shown to oxidize reagents in the hemolysis assay. This results in false positive interference as the oxidized reagents lead to an increase in signal, which is measured as an increase in hemolysis (Dobrovolskaia *et al.*, 2008b).

Another type of chemical interference that NPs may exhibit is the ability to catalyze reactions independent of the enzymes present in the assay. For instance, the LAL assay is an enzymatic method based on the ability of LAL to react with bacterial endotoxin. The assay is used to measure endotoxin contamination for molecules that will be tested *in vitro* and *in vivo*. The three common methods to measure LAL are chromogenic, turbidity, and gel clot. However, NPs with the enzymatic ability to catalyze the LAL reaction in the absence of LAL, such as dendrimers, have the ability to interfere with all three types of readouts (Dobrovolskaia *et al.*, 2008a; Dobrovolskaia, Germolec, and Weaver, 2009).

3.4 NP Surfaces

NP-specific surface interactions can also occur with assay components and include NP surfaces binding assay reagents or trafficking reagents across cellular barriers. As NPs have a larger proportional surface area as opposed to microscale materials, surface factors account for a large part of NP interference and are closely linked to chemical interference in regard to the actual mechanism. Such interference is often difficult to account for as well as to control. Assay reagents or analytes frequently adsorb to the surface of NPs and can cause false positive and false negative results. For example, it has been proposed that NPs may absorb LDH, an assay component commonly used to determine membrane integrity. Binding the enzyme would result in a lowering of the detected enzyme in a sample and indicate less damage to the outer cell membrane than is actually occurring (Stone, Johnston, and Schins, 2009). Gold and polystyrene (PS) NPs have both been found to absorb hemoglobin, also resulting in the NPs appearing less hemolytic than they actually are (Dobrovolskaia *et al.*, 2008b). Multiple NPs, including carbon black and nanotubes (Monteiro-Riviere, Inman, and Zhang, 2009; Worle-Knirsch, Pulskamp, and Krug, 2006) have been found to bind components of the MTT assay, resulting in the NP formulation appearing more toxic to the cells. NPs have also been shown to bind LPS (Dobrovolskaia, Germolec, and Weaver, 2009), a particularly problematic assay interference, as it could potentially give a clean bill of health to an endotoxin-contaminated sample.

NP trafficking may also occur via surfaced interaction. We have reported previously that gold NPs may bind membrane impermeable dyes containing primary amines, such as propidium iodide, and traffic them across the healthy membrane in the absence of membrane damage, producing a false signal for cellular toxicity (Keene *et al.*, 2011). Even more subtle interactions are the reports of NPs depleting cell culture media of nutrients through surface interactions (Kroll *et al.*, 2009; Guo *et al.*, 2008). SWCNTs have been shown to deplete cell culture media of nutrients, causing cytotoxicity when cells were exposed to the depleted media (Casey *et al.*, 2008). Such interference is very difficult to observe or control *in vitro*.

3.5 Unexpected Mechanisms

There are several mechanisms of interference that have been reported in the literature that are specific to a particular assay and do not fall into one of the specific categories mentioned earlier. One example is that dendrimers were found to induce blood clots in a hemolysis assay. These blood clots prevented hemolysis from occurring because of the coagulated blood. Therefore, in this instance, it is important to check for platelet aggregation in addition to the hemolysis measured by an increase in absorbance (Dobrovolskaia *et al.*, 2008b). In addition, NPs may prevent protein–protein binding interactions, which, in turn, will interfere with assays based on antibody binding such as the enzyme-linked immunosorbent assay (ELISA). ELISAs typically measure an upregulation of a particular protein or antigen, such as a proinflammatory cytokine. The measurement involves the binding of a primary antibody to the antigen and the generation of a colorimetric, fluorescent, or luminescent signal created by a substrate that is chemically converted by an enzyme that binds the primary or secondary antibody. Dendrimers were found to interfere with protein–protein binding, preventing the antibodies from properly attaching to their targets (Dobrovolskaia *et al.*, 2008a). In order to measure for this, one could run the assay with the NPs spiked with the antigen to determine whether proper antibody binding occurs. NPs have also been noted to interfere with the materials of the assay. For example, it has been proposed that NPs may interfere with pH electrodes, by absorption to the electrodes,

interference with the solution or gel inside the probes, or creating spurious voltages. Any of these mechanisms of interference could cause inaccurate readings (Casey *et al.*, 2007). Another example is the possibility that NPs may impact the mechanical properties of the agarose gel used during the Comet assay, which could potentially result in erroneous reading of the assay (Handy *et al.*, 2012). It has also been proposed that NPs could interact with the DNA of the Comet assay during electrophoresis, causing nonrepresentative damage (Lin *et al.*, 2009).

4 CONSIDERATIONS FOR ASSAY SELECTION—EXAMPLE: VIABILITY ASSAYS

Selecting the proper end point to use for testing the nanomaterial construct is crucial in order to gain as much robust data out of the testing method as possible. *Viability* assays or *cell toxicity* assays are terms that are often used to describe nanotoxicity assays, but these terms are, in fact, broad terms for a variety of specific assay end points, which warrant further discussion.

Viability assays are an extensive class of assays used in toxicological assessments. In broad terms, viability assays measure some parameter that correlates to a certain function of a cell that can indicate damage and/or repair. For example, examining cells exposed to propidium iodide for fluorescent (excitation: 535 nm and emission: 617 nm) nuclei can indicate the overall membrane integrity of a cell (Lemasters, 2010). MTT assays may indicate the state of mitochondrial activity. There are many different types of functional assays for different cell populations that may all fall under the general *viability* term, including tests for apoptosis and oxidative stress. Therefore, an understanding of what the assay is actually measuring may be beneficial to interpreting both NP toxicity results and NP assay interference. Table 2 lists many of the common viability assays and their mechanism of action. As can be observed from the table, many assays measure the permeability of assay dyes either entering or leaving the cell (such as for propidium iodide or LDH) or the function of mitochondrial enzymes. Both measurements may have inherent problems when applied to NP systems. Membrane permeability assays, for example, have been demonstrated to be invalidated by NP trafficking dye molecules across intact membranes (Keene *et al.*, 2011) or NPs

Table 2. Common viability assays.

Common name	Mechanism	Common applications	Endpoint	Common issues
MTT	Reduction of compound to colored formazan derivative	Cell proliferation, mitochondrial function, apoptosis	Absorbance 500–600 nm	Colorimetric, oxidize/reduce
XTT	Reduction of compound to colored formazan derivative	Viability, cell proliferation, mitochondrial function, apoptosis	Absorbance 450–500 nm	Colorimetric, oxidize/reduce
MTS	Reduction of compound to colored formazan derivative	Viability, cell proliferation, mitochondrial function, apoptosis	Absorbance 490–500 nm	Colorimetric, oxidize/reduce
Alamar blue® Cell Titer Blue®	Reduction of compound to fluorescent formazan derivative	Viability, cell proliferation, mitochondrial function, apoptosis	Fluorescence Ex: 579 nm/Em: 584	Quenching oxidize/reduce
WST	Reduction of compound to a colored formazan derivative	Cell proliferation, cell viability cytotoxicity	Absorbance 440 nm	Colorimetric, oxidize/reduce
Propidium iodide	Fluorescence dye, with emission enhanced and shifted upon intercalation between DNA bases. Generally excluded from viable cells	Membrane integrity, apoptosis, live/dead assays	Fluorescence Ex 535/Em 617 nm	Trafficking
LDH	Oxidation of lactate to pyruvate coupled to the reduction of a compound to a colored product LDH is a cytosolic enzyme that may be released into cell culture medium when the cell membrane is damaged	Membrane integrity, apoptosis cell proliferation	Absorbance 492 nm	Colometric, NP absorption, quenching
LDH/CytoToxOne homogeneous membrane integrity assays®	Oxidation of lactate to pyruvate coupled to the reduction of a compound to a fluorescent product. LDH is a cytosolic enzyme that may be released into cell culture medium when the cell membrane is damaged	Membrane integrity, apoptosis cell proliferation	Fluorescence Ex: 560 nm/Em: 590 nm	Colometric, NP absorption, quenching
Neutral red (basic red 5, toluylene red)/TOX-4®	Viable cells take up dye by active transport	Cell proliferation	Absorbtion 539–544 nm	Colormetric, NP absorption, pH sensitive quenching
Trypan blue (azidine blue 3B, benzamine blue 3B)	Generally excluded by viable cells	Membrane integrity, cell proliferation	Usually visual analysis (dye stains dead cells)	Colormetric, NP agglomerates, counted as cells, trafficking

(continued overleaf)

Table 2. (Continued)

Common name	Mechanism	Common applications	Endpoint	Common issues
Methylene blue	Generally excluded by viable cells	Membrane integrity	Absorbance 664 nm	Colometric trafficking
Calcein AM	Compound crosses cell membrane and is hydrolyzed by esterases into membrane impermeant calcein by live cells	Cell Proliferation, viability	Fluorescent Ex: 495 nm/Em: 516 nm	Quenching blocking of fluorescent signal, enhancement of fluorescent signal
Live/Dead® calcein AM/ethidium homodimer 1	Calcein AM crosses cell membrane and is hydrolyzed by esterases into membrane impermeant calcein by live cells. Ethidium homodimer-1 is generally excluded by viable cells and has enhanced fluorescence upon binding to nucleic acids	Live/dead assays	Calcein AM Fluorescent Ex: 495 nm /Em: 516 nm Ethidium homodimer-1 Fluorescent Ex: 528 nm/Em : 617 nm	Quenching blocking of fluorescent signal enhancement of fluorescent signal trafficking
Live/dead calcein AM/propidium iodide	Calcein AM crosses cell membrane and is hydrolyzed by esterases into membrane impermeant calcein by live cells Pi is generally excluded by viable cells and has enhanced fluorescence upon binding to nucleic acids	Live/dead assays	Calcein AM Fluorescent Ex: 495 nm/Em: 516 nm Pi fluorescence Ex: 535 Em: 617 nm	Quenching blocking of fluorescent signal enhancement of fluorescent signal trafficking
CyQuant®	Fluorescence dye, with emission enhanced upon binding to cellular nuclei acids. Amount of DNA correlates to cell number	Cell proliferation	Fluorescent Ex: 485 nm/Em: 530 nm	Quenching
Thymidine incorporation assay	³ H-thymidine is incorporated into new strands of chromosomal DNA during cell division	Cell proliferation	Scintillation counting	—
Bromodeoxyuridine (BrdU)	Thymidine analog detected during DNA synthesis using immunohistochemistry techniques	Cell proliferation	Detection with anti-BrdU antibody	Quenching
Fluorescein diacetate (FDA)	FDA is hydrolyzed by intracellular enzymes to fluorescein	Enzymatic activity, membrane integrity	Fluorescent Ex: 494 nm/Em: 521	Quenching
ATP/CellTiter-Glo/flash assay	Luciferin is catalyzed by luciferase, ATP, Mg ²⁺ , and molecular oxygen to oxyluciferin. Luminescence is correlated to ATP	Cell proliferation	Luminescent	Quenching

The table lists common viability assays, their mechanism of action, and common NP interference issues.

binding LDH and significantly reducing the assay signal (Kroll *et al.*, 2009; Clift *et al.*, 2008; Suska *et al.*, 2005).

Mitochondrial function based on the reduction of MTT is one of the more popular viability assays. There are multiple derivations of this assay, including the water-soluble derivatives XTT and MTS (Buttke, McCubrey, and Owen, 1993). MTT is reduced to a purple formazan product by mitochondrial reductase. Therefore, it is often used to study mitochondrial function (although other areas in the cell have also been shown to be able to reduce MTT compounds) (Bernas and Dobrucki, 2002). Metabolic activity, cytotoxicity, viability, and cell proliferation have all been used as end points for this compound (Mosmann, 1983). All of the assays are based on the reduction of a parent compound to a colored product. Therefore, any NP that functions as a reducer may interfere with this assay. In addition, MTT-based assays have been shown to cause other interference, including NP colorimetric interference (Kroll *et al.*, 2011), NPs binding MTT product (Monteiro-Riviere, Inman, and Zhang, 2009; Worle-Knirsch *et al.*, 2006; Monteiro-Riviere *et al.* 2006), NPs reducing MTT (Laaksonen *et al.*, 2007; Berg *et al.*, 2010; Belyanskaya *et al.*, 2007), NPs preventing MTT reduction (Kroll *et al.*, 2011), and NPs interfering with efflux pumps and preventing MTT from entering cell (Murthy *et al.* 2009).

Owing to the severe, and sometimes subtle, interference with standard kit viability assays, it is best to list the physiological function that the assay is measuring, rather than simply “viability.” This distinction helps focus the decision of appropriate controls, interpretations, and complementary testing, if needed. A second recommendation to aid in correctly interpreting NP viability assay results is to visually examine the cells with a light microscope (Lewinski, Colvin, and Drezek, 2008). This quick check can confirm that an assay that reports healthy cells are indeed viable and vice versa. Finally, multiple tests checking multiple functional end points will help detail assay interference as well as begin to elucidate mechanisms of toxicity, if any.

Viability end points are just one type of common assay end point that may be measured. Immunotoxicity, genotoxicity, oxidative stress, and sterility/pyrogenicity testing all may be measured via high-throughput *in vitro* assays. For each assay class, it is important to consider the actual end point

and chemistries involved in the assay as well as possible NP interference.

4.1 Considerations When Setting up the Assay

Once the external/physical factors are taken care of, and potential assay interference has been discussed, some additional considerations should be considered when setting up the actual assay.

4.1.1 Choice of Cell Types and Cell Culture Conditions

A variety of human and rodent cell types have been employed for NP cytotoxicity testing *in vitro*. As the ultimate aim of cytotoxicity studies is to be applicable and correlative with *in vivo* findings, the type of cells used *in vitro* should be related to the *in vivo* model. For example, lung macrophages, fibroblasts, or epithelial lung cells are used for lung inhalation studies (Han *et al.*, 2012), hepatocytes are used for liver toxicity testing (Ahmad *et al.*, 2012), keratinocytes are used in case of skin studies (Yin *et al.*, 2012), and intestinal epithelial cells are used for oral NP delivery models (Gerloff *et al.*, 2012), to name only a few. While primary cell lines would often be the preferred choice of cells to use for *in vitro* assays, limited availability (in the case of human cells), variability from one experiment to another (e.g., cell differentiation from bone marrow cells), and long isolation and purification times often mean that immortalized cell lines are used instead (Jones *et al.*, 2009). Cell lines are commercially available and, in most of the cases, are well characterized.

While most of the reported cytotoxicity studies employ one cell line, there is a growing consent that several cell types (and end points, as discussed earlier) are required to determine whether or not NPs are cytotoxic. Kroll *et al.* (2011) showed that when comparing reactive oxygen species formation in 10 different cell lines exposed to 23 different types of NPs there are differences not only among cell types but also among cells lines that were derived from same organ (bronchial vs alveolar epithelial cells or kidney cell lines). The variability between different cell lines/cell types could be associated with their phagocytic (or lack of) ability, metabolic status, culture conditions, cell cycle, and so on. For

example, A459 human lung carcinoma cells incorporated more NPs during G2/M phase of the cell cycle and the least during G0/G1 phase (Kim *et al.*, 2011). Exposure of synchronized cultures (grown in media without FBS) to NPs and media with FBS will increase the rate of division and therefore the incorporation of NPs (Kim *et al.*, 2011).

The majority of cell types used (as listed earlier) are adherent cells and in cytotoxicity assays, in general, cell cultures are exposed to NPs once they reached confluency (e.g., 70–80%). However, the majority of scientific articles do not record the precise number of cells used for cytotoxicity assays. Although all cell types used in one experiment are confluent (e.g., 80%), there might be differences in cell numbers because of differences in cell size or surface area covered by cells. Differences in cell responses can also arise because of the phenotype (adherent vs nonadherent) of the cells. During culture, NPs will sediment and settle on cell surface, which might influence the amount or rate of NP uptake, especially in the case of adherent cells. On the other hand, blood cells (monocytes, lymphocytes, or platelets) are cultured in suspension in which case the NP/cell interactions might be vastly different than during adherent conditions. In order to avoid sedimentation and NPs settling on the cell surface, inverted cell cultures were introduced by Cho, Zhang, and Xia (2011). It was shown that when human breast adenocarcinoma cell lines were cultured in inverted conditions, NP uptake was significantly decreased for a variety of Au NPs compared to traditional, upright cell culture conditions.

4.1.2 NP Dosimetry

As discussed earlier, NPs contain unique physical properties that lead to NP agglomeration/aggregation, sedimentation, and diffusion, which may ultimately modify NP uptake and cellular doses. Therefore, when designing toxicity experiments, not only the concentrations used, but also the NPs characteristics should be evaluated. Dynamic light scattering (DLS) measurements coupled with transmission electron microscopy (TEM) observations in culture media will indicate whether or not agglomerates/aggregates are formed and the size range of the resulting structures. These are important features as it was shown that Au NP distribution *in vivo* is size and aggregation/agglomeration dependent (Keene

et al., 2012). Agglomerates/aggregates should be considered especially in *in vitro* time course studies as they tend to sediment faster compared to primary particles. Another factor that will influence NP sedimentation rate is the culture media volume/depth. Different results might be generated when cells are cultured in a 6-well plate (e.g., 2 ml/well) versus 48-well plates (e.g., 0.2 ml/well). In this case, differences in cell responses might arise because of sedimentation or diffusion rates. Considering the physicochemical characteristics of NPs, Hinderliter *et al.* (2010) have developed the *in vitro* sedimentation, diffusion, and dosimetry (ISDD) model to determine cellular doses and predict NP toxicity. The ISDD model was tested *in vitro* using different sizes of polystyrene NPs, SiO₂ and iron oxide NPs, and the authors concluded that the ISDD model prediction was similar (within twofold difference) to cellular doses obtained *in vitro*. Therefore, the ISDD model can be adapted to determine cytotoxicity of NPs, especially in cases where *in vitro* or *in vivo* data are lacking.

Considering the unique properties of NPs, determining the cellular dose of NPs (how many NPs are incorporated per cell) is a cumbersome task. As a starting point, when determining NP concentrations for cytotoxicity assays, it is helpful when the concentrations are expressed not only in mass concentrations but also as NP number concentration and NP surface area concentration. The following example demonstrates the complexity of determining NP dose and corresponding toxicity end points. In a study by Hussain *et al.*, cytotoxicity end points from multiple *in vitro* assays were reported for CdO NPs (1 μ m), Ag NPs (15 and 100 nm), and MoO₃ NPs (30 and 150 nm) using nominal media mass concentrations (μ g ml⁻¹). It was determined that CdO NPs were 2.5- to 4-fold more toxic compared to Ag NPs or MoO₃ NPs when exposed to BRL (buffalo rat liver) 3A rat liver cells (Hussain *et al.*, 2005). Using the same data set, Teeguarden *et al.* (2007) reevaluated the above-mentioned results, this time accounting for NPs' gravity and diffusion through the media and using surface area concentration (cm² ml⁻¹), rather than the media mass concentration. The authors found that the cytotoxicity of all of the NPs was within a twofold difference for the reported toxicity end points.

Waters *et al.* (2009) compared the cytotoxicity profile of six types of silica NPs (7–300 nm) and calculated the LC₅₀ (concentration where 50% viability

is expected at 24 h) expressed in mass concentration ($\mu\text{g ml}^{-1}$), NP number concentration, and surface concentration ($\text{cm}^2 \text{ml}^{-1}$). When concentration of NP is expressed as mass concentration, there was more than an order of magnitude difference in LC_{50} between the NPs with the smallest and the largest diameters ($20 \mu\text{g ml}^{-1}$ for SiO_2 NP 7 nm compared to $\sim 600 \mu\text{g ml}^{-1}$ for SiO_2 NP 300 nm). When the concentration is expressed as total NP, number data shows that in case of NPs with larger diameter, fewer NP numbers are required to induce toxicity (10^{10} particles ml^{-1} for SiO_2 NP 300 nm and 10^{14} particle ml^{-1} for SiO_2 NP 7 nm). However, when concentration was expressed as total surface area, the LC_{50} was $85 \text{ cm}^2 \text{ml}^{-1}$ for all SiO_2 NPs (independent of the diameter). In this case, the authors found that NP surface area was more informative in determining LC_{50} values and the functional changes showed a tighter correlation with surface area rather than with mass concentration or NP number. NP number can be calculated based on weight, diameter, and NP density. Furthermore, the ratio between NP number and cell number can also be calculated in which case, as discussed earlier, the precise cell number should be recorded.

Although the end point in cytotoxicity assays is evaluation of cell viability, it is equally important to determine if NP incorporation occurred and, if possible, how much of the NP dose was incorporated into cells. In order to identify NP incorporation, several techniques (confocal and fluorescent microscopy, transmission and scanning electron microscopy, and CytoViva[®] with hyperspectral imaging) are used; however, these techniques often offer only a semiquantitative, at best, evaluation of NP incorporation. In the case of fluorescent-labeled NPs (for example, PS NPs), incorporation and NP detection are facilitated by the fluorescent dye, and cellular uptake can be analyzed on a flow cytometer (and expressed as fluorescence intensity) and a fluorescent microscope (to ensure that NPs are localized inside the cell and not on the cell surface). Using fluorescein isothiocyanate (FITC)-conjugated PS NPs, Lunov *et al.* (2011) demonstrated that the NP uptake, kinetics, and mechanism are different between human macrophage cell lines and primary cells, presence and absence of serum in the culture media, and carboxy- and amino-terminal PS NPs. For example, human macrophage cell lines incorporated ~ 4 times more carboxy-terminal PS NPs

compared to monocytic human cells (Lunov *et al.*, 2011).

Owing to Au NP versatility, there are several methods developed to quantify Au NP concentration in solution and Au NP cellular uptake. Aggarwal and Dobrovolskaia (2011) developed an elegant fluorescent method where fluorescence develops on complex formation between Au(III), ligand, and an acidic dye; therefore, fluorescence signal is proportional with Au NP concentration and can be easily quantified. This assay can be used to determine Au NP concentration in solution as well as in cell lysates. One of the most sensitive methods to determine NP concentration and uptake is mass spectrometry (MS). Laser desorption/ionization MS was employed to determine cellular incorporation of two types of Au NPs with readily ionizable surface functionalities in the same cell lysate of monkey kidney cells (Zhu *et al.*, 2008); inductively coupled plasma MS and UV-vis spectroscopic method were used to determine cellular uptake of Au NPs in inverted versus upright cultured cells (Cho, Zhang, and Xia, 2011); and inductively coupled plasma atomic emission MS was used to determine the uptake half-life of Au NPs (Chithrani *et al.*, 2006).

Cellular uptake of NP not only serves quantification purposes but also offers the possibility to evaluate NP uptake kinetics (rate, time, peak, and incorporated NPs vs NPs in culture media), determining the influence of culture conditions or cell type on NP uptake as well as the maximum NP “load” inside the cells that causes cytotoxicity. These values can be expressed in NP mass concentration, number of NP/cell, or total NP surface area.

Regardless of the concentration units used, it is important to test relevant applicable concentrations. Although as proof of concept a low and a high concentration are often used in cytotoxicity studies, it is often a common practice to “overload” the cell cultures *in vitro* ($100 \mu\text{g ml}^{-1}$ or even higher concentrations). When attempting to use *in vitro* assays as a predictor of *in vivo* performance, the results of these overloaded assays may not be applicable. In some cases, previous *in vivo* work for similar classes of NPs may guide *in vitro* dosage selection. For example, previous distribution and *in vivo* toxicity studies for AuNPs (Cho *et al.*, 2009; De Jong *et al.*, 2008; Lasagna-Reeves *et al.*, 2010; Keene *et al.*, 2012) may also be used to determine how many NPs localize in a particular organ/tissue to recalculate relevant dosing schemes in an *in vitro*

setup. Certainly, the distribution and localization of an NP will vary depending on size, core, coating, agglomeration, administration route, and so on but, nevertheless, these *in vivo* studies, as well as intended in-life applications and doses, may offer a starting point in determining relevant concentration range to be used *in vitro*.

4.2 Controls and Running of the Assays

In all cases, when applying *in vitro* assays to NPs, it is imperative to run appropriate controls. At a minimum, a no cell control, with the NPs exposed to all other assay conditions, will highlight many obvious forms of interference. Additional controls include positive and negative controls, where compounds known to produce an either positive or negative response are included in the assay setup. Another common control is the NP-spiked positive control. There are two methods to complete this control. One method is to reserve wells for a positive control to the assay spiked with NPs for the entire assay time course. This control would establish whether the NPs interfere with any mechanism of the assay to prevent the visualization of a positive signal [comparing the spiked positive control with the positive control has also been termed *inhibition/enhancement control* (Dobrovolskaia *et al.*, 2010)]. One possible problem with this method is that a decrease in the signal of the positive control could be caused because the NPs may actually prevent the positive control from causing toxicity. A second method is a postspiking NP positive control where the plate is read as usual and then NPs are added immediately after the reading to the positive control wells and the plate reread to monitor for absorbance/fluorescence/luminescence changes. This control would be useful to help probe for either enhancement or quenching of a fluorescent or luminescent signal by the NPs. However, this control would only be useful for measuring interference with the readout mechanism, not for measuring other types of interference caused by the NPs such as trafficking. Additional controls include evaluating the NP solvent and NP excipient and/or stabilizer for toxicity as compared to the “no treatment” control. Such a control is termed *formulation or filtrate/retentate control*.

In addition to any controls, the cells should always be visually examined under a light microscope to

identify obvious signs of positive or negative interference, such as an assay that measures 100% viability when all of the adherent cells have detached. If measuring cell proliferation, it is also helpful to initially also manually count the cells with a hemocytometer, as these results are far less susceptible to interference by NP constructs.

In order to test for sources of chemical interference in cell assays, the easiest method is to combine all components of the assay without the presence of cells or cellular component (i.e., no cell control). If this type of interference is detected, it is unlikely that the assay will be able to be used. Therefore, the recommendation is to choose an assay that uses a different mechanism to measure the same effect. In many cases, along with visual inspection, when applicable, using two methods to test for the same end point would be the first line of defense to account for NP assay interference that is difficult to detect.

As discussed earlier, NP interference should be expected with all common *in vitro* toxicity assays. However, there are controls and methods that can be used to help detect and overcome some of the most common mechanisms of interference. Some of these controls are listed in Table 3. Note that although multiple controls are needed initially for new NPs and assays, once the assay is validated, some of these controls may be eliminated. While these controls will not uncover or eliminate all mechanisms of interference, they will catch the most obvious forms of interference. Once the mechanism of interference is identified, one may try to compensate for the interference in order to produce valid results. In some cases, such as when turbidity is causing an elevated baseline, the absorbance/fluorescence/luminescence of the NPs may be subtracted (Monteiro-Riviere, Inman, and Zhang, 2009; Keene *et al.*, 2011; Ying and Hwang, 2010) (although it is preferable to use an assay that has no interference issues, in some cases, this may not be feasible). Another mechanism to account for interference is to centrifuge the assay materials to remove the NPs before measurement, which may prevent absorbance, fluorescence, and luminescence interference that is directly due to the presence of the NPs (Oostingh *et al.*, 2011). However, this will only work for certain types of assays, the assay molecules cannot bind to the NP surface, and the NPs must be large/dense enough to be easily concentrated by centrifugation.

Table 3. List of common NP assay controls and functions.

Control	Method	Function
No cell	Assay is run without cells	Allows for identification of chemical and readout NP interference mechanisms
No treatment (also termed <i>negative control</i>)	Cells are not treated with NP	Serves as a baseline for normal cell conditions
No catalyst	Assays is run without necessary catalyst for <i>in vitro</i> reaction	Measures NP's own ability to catalyze reaction
Positive	An agent is used to produce a robust assay response	Ensures that the assay is working as expected
Positive-spiked (also termed <i>inhibition/enhancement</i>)	The NP is spiked into well containing a positive control	Probes for NP inhibition/enhancement of assay signal
Positive-spiked POST-run	The NP is spiked into well after the initial assay is read	Probes for optical interference of NP with assay
Solvent	The solvent used to disperse the NPs is added to wells at the same concentration/volume of the NP formulation	Ensures that any observed effects arise from the NP instead of the solvent
Formulation (also termed <i>filtrate/retentate</i>)	All components of the formulation minus the NP are tested	Ensures that any observed effects arise from the NP as opposed to other aspects of the formulation
Visual inspection	Cells are visually inspected with a light microscope after the assay is performed	Mainly used for toxicity assays, the visual confirmation allows for a cross check of toxicity results
Multiple assays	More than one assay is used for the same endpoint	Serves as cross-check validation for toxicity endpoint

5 CONCLUSION/SUMMARY

With the expanding application of nanotechnology into the medical field, a great variety of NPs will need to be analyzed for toxicity and other biological responses. Often, the first line of testing will be *in vitro* assays. Although these tests are popular because of their ease of use, specific considerations must be considered when applying them to NPs. In particular, assay selection, setup, and detection/mitigation of assay interference play a crucial role in obtaining robust data from *in vitro* assays. By understanding the assay end points and designing appropriately controlled experiments, these assays may be used for a variety of NP applications.

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In Vivo Evaluation of Acute and Chronic Nanotoxicity

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1 INTRODUCTION

The global nanotechnology market value, which was worth 300 billion in 2011, is expected to increase by fivefold by the end of 2013 and by more than 100-fold by the year 2041 (Antunes *et al.*, 2013). Nanotechnology is a rapidly evolving field and is set to play a pivotal role in various industry segments, medicine, and biotechnology. The emergence of nanomedicine in recent years has led to the development of new materials for controlled and targeted drug release as well as new methodological approaches for tissue engineering, treatment, and diagnosis of a variety of diseases. Nanomedicine has historically focused on therapeutic drug delivery or diagnosis of cancer (see Chapter 10) (Kawasaki and Player, 2005). However, new applications are emerging such as vaccine adjuvants, medical imaging, and gene therapy (Christensen *et al.*, 2007; Cormode *et al.*, 2009; Bharali *et al.*, 2005; Krebs *et al.*, 2010). A recent literature survey identified 363 nanomedicines already approved or in the clinical phase, which were used in 789 clinical trials (Etheridge *et al.*, 2013).

The unique properties of nanomedicines enable an unprecedented opportunity for manipulating biochemical interactions, tissue targeting, and barrier crossing and also pose a problem for understanding the novel risk of toxicity. The toxicology data on the persistence of degradation products of

nanomedicines are sparse, mainly because the field is still nascent. For medical approval, nanomedicines are subject to the same rigid preclinical, clinical, and post-release regulations as conventionally sized drugs. However, most Food and Drug Administration (FDA) regulations were written before the era of nanotechnology (Ventola, 2012). The majority of current nanomaterial regulation outlines closely resemble regulations developed for standard materials. Traditional milligram/kilogram dose measurements still need to be investigated in order for comparison to other medicines and poisons but fall short of comprehensive measurement of nanomedicine exposure (Chan, 2006). In the United States, nanomaterials may, at the discretion of regulatory bodies, be deemed to fall under toxic substances regulations, whereas in the European Union, they may fall under the regulation of the “Registration, Evaluation, and Authorization of Chemicals Act” (Gwinn and Tran, 2010).

Regulatory agencies such the European Medicines Agency (EMA) and the FDA have recognized the specificity of the safety issues of nanomedicines and have begun to implement a distinctive program required for their approval (Ventola, 2012). A battery of characteristics needs to be considered such as surface area, size, charge, porosity, persistence of by-products, and the interaction of nanomaterials with proteins and molecules in living organisms (see Chapter 2). Unfortunately, there is currently

no satisfactory standardized and agreed upon protocol that is specific to the safety of nano-sized materials.

The increasing diversity of engineered nanomaterials used for medical applications such as metals, dendrimers, chitosan, silica, and carbon nanotubes (CNTs) has raised concerns regarding the safety of nanoparticles as each construct may have distinct toxicity profile that is modulated by physicochemical characteristics or the route of exposure (Fadeel and Garcia-Bennett, 2010). *In vivo* evaluation of nanotoxicity remains the most reliable approach to determine nanotoxicity compared to both *in vitro* and *in silico* methods. Standard *in vitro* tests to evaluate toxicity are not always applicable to nanotechnology. *In vitro* methods do not consider the specific biological attributes of nanoconstructs such as biodistribution, metabolization, excretion, and the overall influence on the immune system, all of which are specific to multiorgan interactions particular to *in vivo* testing.

In this chapter, we summarize the current knowledge related to the *in vivo* safety of common nanomaterials. We also discuss the influence of specific exposure routes on nanotoxicology and provide practical guidelines for testing acute and chronic nanotoxicity *in vivo*.

2 CHARACTERIZATION OF NANOMATERIALS

Nanomaterial characterization is the most essential factor when considering nanomaterial toxicity and cannot be assumed to be identical to the bulk material (Jiang *et al.*, 2008). Although some material properties, such as chemical composition or crystal structure, are similar, there is a great difference in the biological interaction of bulk materials and nanomaterials because of different physical characteristics such as size, shape, surface area, porosity, and reactivity (Sayes and Warheit, 2009). Thus, understanding the characteristics of the nanoparticle becomes crucial in order to correlate the observed effects *in vivo*. In addition, characterization data is important to facilitate comparison of toxicity results of a given nanoparticle with other nanoconstructs. With recent advances in instrument technologies, it is possible to precisely measure the physicochemical properties of nanoparticles. Dynamic light scattering, scanning electron microscopy, and

transmission electron microscopy techniques can be used to establish the size and shape of constructs. X-ray photoelectron spectroscopy can measure the chemical composition. Mass spectrometry can conduct mass and elemental analysis and spectroscopy techniques can be utilized to measure absorption, emission, or scattering. Evaluating the purity of nanomaterials is essential before assessing their toxicity so that any nonspecific toxicity attributed to material impurities or biological contaminations can be excluded. Chromatographic techniques such as high pressure liquid chromatography or size exclusion chromatography can indicate the presence of impurities in the sample. Some of the techniques mentioned earlier, such as mass spectrometry and spectral analysis, also provide valuable information regarding contamination or impurities. Endotoxins or lipopolysaccharides (LPS) are small, bacterially derived hydrophobic molecules that can easily contaminate nanomaterials. The presence of LPS can considerably influence *in vivo* toxicity because of their pronounced biological effects. The commonly used FDA-approved techniques for endotoxin detection are the rabbit pyrogen test and limulus amebocyte lysate (LAL) assay (Greish, Thiagarajan, and Ghandehari, 2012).

In recent years, a vast array of newly engineered and rediscovered nanomaterials has been used to synthesize nanomedicines. Liposomes, dendrimers, conjugates, and polymeric micelles are commonly used for controlled drug delivery to cancer tissue and constitute a large proportion of the clinical trials currently being undertaken (Etheridge *et al.*, 2013). However, only liposomes and conjugates are approved for clinical cancer treatment (Taurin, Nehoff, and Greish, 2012). Following are some examples of toxicological problems attributed to nanoconstructs being investigated for use in the medical field.

2.1 Liposomes

Liposomes are spherical constructs composed primarily of lipid, which are often utilized for drug and gene delivery as well as for diagnosis (Kshirsagar *et al.*, 2005, Sonoke *et al.*, 2011). Their biocompatibility, biodegradability, ability to encapsulate hydrophilic and lipophilic drugs, and the ability to functionalize the surface with targeting moieties have resulted in this construct becoming the

commonly researched for medical purposes. As a result, several liposomes have been approved by the FDA for clinical use such as Doxil, Danuxome, Myocet, Depocyt, and MEPACT (Food and Drug Administration, 1996; Batist *et al.*, 2002; Gordon *et al.*, 2001; Chhikara and Parang, 2010; Ando *et al.*, 2011). Liposome physicochemical properties such as size and charge differ considerably depending on their lipid composition and method of preparation (Akbarzadeh *et al.*, 2013). This is a crucial factor when pursuing liposomes for medical use as the uptake of liposomes by macrophages of the reticuloendothelial system (RES) is highly dependent on the liposomes size and charge (Senior and Gregoriadis, 1982; Senior *et al.*, 1991). Excess uptake of liposomes and subsequent lipid deposition in cells of the RES can result in alteration of the cells' phagocytic capacity and gene expression, and may down regulate NO and tumor necrosis factor- α ((TNF)- α) production by macrophages (Filion and Phillips, 1997); however, no evidence has been found to suggest that therapeutic doses of liposomes used for medical purposes can induce immunosuppression *in vivo* (Szebeni and Moghimi, 2009). Liposomes have also shown the ability to cause complement activation, resulting in the potentially lethal adverse effect known as *pseudoallergy* (Szebeni, 2001). Pseudoallergy is further discussed in the special tests section.

2.2 Polymer Conjugates

Early development of polymeric nanomedicines, such as styrene maleic acid-conjugated neocarzinostatin (Maeda *et al.*, 1985), has prompted the emergence of polymer-based nanomedicine. Synthetic polymers such as N-(2-hydroxypropyl) methacrylamide (HPMA) copolymers, poly(ethyleneimine) (PEI), divinyl ether and maleic anhydride (DIVEMA), polyethylene glycol (PEG), and natural polymers such as chitosan have all been used at the clinical level as polymeric platforms for drug delivery (Duncan, 2003). The degradation of these polymers is an important consideration as nonbiodegradable carriers can pose significant risk. Unless the nanocarrier is biodegradable, it will remain in the body and be dealt with as a foreign body. The innate elements of the immune system can be stimulated nonspecifically by these foreign bodies through receptors such as TLR-4

(Kedmi, Ben-Arie, and Peer, 2010). Activated macrophages will phagocytose and attempt to degrade the nanocarrier. The failure to do so may lead to the formation of foreign body giant cells caused by fusion of multiple macrophages or monocytes (Anderson, Rodriguez, and Chang, 2008) and ultimately to the formation of lesions resembling granulomas (Mukhopadhyay and Gal, 2010). This can potentially result in the pathological formation of a dense fibrous capsule replacing the original functional tissue. Another concern in relation to the accumulation of nondegradable materials is the induction of malignancy resulting from frustrated phagocytosis and prolonged inflammation (O'Neill, 2008).

Click chemistry, the combination of small units using a simple, stereospecific protocol, has become increasingly popular and promoted widespread utilization of polymers (Hein, Liu, and Wang, 2008). The control over subunit addition results in a predictable chemical structure allowing the control of its degradation. Malkoch *et al.* have demonstrated the use of click chemistry to tune the degradation of PEG-based hydrogels (Malkoch *et al.*, 2006). This approach presents a possible option to overcome problems associated with degradation of polymers used for nanomedicine.

2.3 Dendrimers

Dendrimers have gained a lot of interest over the last few years as drug carriers because of their well-defined polymeric branching structure, their low polydispersity index, and their functionalized surface diversity. Although their small size (≤ 10 nm) limits drug incorporation into the dendrimers, their large surface area allows drug binding to the outer branches (Svenson and Tomalia, 2005). Dendrimers have been developed to improve drug delivery for the treatment of various cancers and, more recently, for the treatment of prion disease, Alzheimer's disease, as antiviral and antibacterial agents and for tissue repair (Gajbhiye *et al.*, 2009). Unfortunately, only two compounds have reached clinical development, namely, the contrast agent Gadomer[®] in phase II clinical trials, used for the delineation of regions of microvascular obstruction (Barkhausen *et al.*, 2003), and the topical virucide VivaGel[®], currently undergoing a phase I clinical trial (NCT00442910) intended for intravaginal application to prevent HIV transmission (O'Loughlin *et al.*, 2010).

The *in vivo* toxicity of dendrimers is largely modulated by their functional groups as these are the structures that come into contact with the tissues of the exposed organism. For example, amine-terminated PAMAM dendrimers showed higher toxicity compared to the carboxyl- and hydroxyl-terminated PAMAM dendrimers (Greish *et al.*, 2011). Amine-terminated dendrimers triggered hemotoxicity in the form of a life-threatening disseminated intravascular coagulation-like condition (Greish *et al.*, 2011), whereas carboxyl- and hydroxyl-terminated PAMAM dendrimers were tolerated even when the dose exceeded that of the amine-terminated dendrimers 50-fold.

2.4 Carbon-based Nanomaterials

CNTs can be engineered as single-walled carbon nanotubes (SWCNTs) or multi-walled carbon nanotubes (MWCNTs). CNTs have shown promise in the areas of drug delivery, photothermal therapy, and tissue engineering (Firme and Bandaru, 2010). CNTs are also increasingly being used in industrial products such as car tires and nanoprobe, which have resulted in the global market value of CNTs reaching several billion dollars (Venkata Ramana, Yu, and Seshiah, 2013). CNTs are thought to be highly biopersistent, which increases the risk of environmental exposure and subsequently toxicity. The toxicity induced by exposure to CNTs is also highly dependent on the CNTs structure and purity.

Asbestos is an established carcinogen that induces the formation of granulomas, which progress to mesothelioma, a rare form of cancer. The induction of these tumors is thought to be a consequence of the long, filamentous nature of asbestos, which prevents macrophages from effectively phagocytosing and degrading the fiber, termed *frustrated phagocytosis*. This frustrated phagocytosis leads to inflammation and formation of granulomas, which may progress to mesothelioma. Evidence that this is a consequence of structural and not chemical characteristics is apparent when considering that both silver nanowires and CNTs of sufficient length have been observed to induce frustrated phagocytosis (Schinwald and Donaldson, 2012; Poland *et al.*, 2008). CNTs are generally engineered as high aspect ratio filaments that bear a strong structural similarity to asbestos fibers and have previously

been shown to induce asbestos-like inflammation and foreign body granulomas in mice when injected into the intraperitoneal cavity in order to model the pleural cavity (Poland *et al.*, 2008). Moreover, the purity of the CNTs under investigation is unlikely to be the sole cause. Intratracheal instillation of raw CNTs (26.9% iron), purified CNTs (2.14% iron), and CarboLex nanotubes (0.53% iron) at 0.5 mg caused granulomas in 100% of animals treated (Lam *et al.*, 2004). It is important to note that unlike asbestos, evidence for these granulomas progressing to mesotheliomas has not yet been found (Varga and Szendi, 2010).

2.5 Silica Nanoparticles

Mesoporous silica nanoparticles (MSNs) have been developed for multiple biomedical applications such as biosensors, DNA delivery, and controlled drug delivery (Benezra *et al.*, 2011; Rosenholm, Sahlgren, and Linden, 2010a; Rosenholm *et al.*, 2010b; Popat *et al.*, 2011). The FDA has approved the first clinical trial of silica nanoparticles in humans for Cornell dots, multimodal silica nanoparticles that enclose dye molecules to detect cancer cells (Choi *et al.*, 2007). MSNs are attractive nanoconstructs as it is possible to achieve a predetermined size and shape with a large surface area, large pore volume, and ability to functionalize the surface of the nanoparticle with a vast range of organic functional groups (Qhobosheane *et al.*, 2001; Yang, Gai, and Lin, 2012).

Although a large surface area and pore size make MSNs desirable for the purposes of drug and dye loading, the same characteristic also serves as a potential factor to increase toxicity and limit the dose that may be safely administered. Comparison of silica nanoparticles with and without pores showed that porosity was a major determining factor when considering the toxicity of the nanoparticles (Greish *et al.*, 2011). MSNs caused renal congestion, resulting in a maximum tolerated dose (MTD) 15-fold lower than that of solid silica nanoparticles (Greish *et al.*, 2011) that may potentially limit the utilization of these constructs in the clinic as, with the reduction in pore number, the ability to load drugs is also reduced.

Similarly, although the ability to functionalize the surface of silica nanoparticles is considered a boon for medical use, it also serves as a possible

mechanism for induction of cytotoxicity. Proteins readily adsorb to the surface of silica nanoparticles resulting in extensive nonspecific uptake into organs such as the liver where the nanoparticles cause inflammation, silicotic nodule-like lesions, and fibrosis (Liu *et al.*, 2012). There is indication that protein adsorption onto the surface of the silica nanoparticles may increase cytotoxicity (Dutta *et al.*, 2007) and that silica nanoparticles adsorb fibrinogen (Hemmerlé *et al.*, 1999); however, the importance of protein adsorption on *in vivo* toxicity has not yet been established.

2.6 Metal-based Nanoparticles

Metal-based nanoparticles such as gold, silver, iron, zinc, and titanium are currently being developed for diverse applications such as cosmetics, diagnosis, antibacterial treatment, and drug delivery.

Particular concerns about metal-based nanoparticles involve their persistence and reactivity. Many metal nanoparticles have been demonstrated to have long retention times *in vivo*. For example, gold nanoparticles (Au-NPs) injected into adult male Wistar rats showed an increase in the liver concentration from 1 and 2 months post-injection of 0.01 mg kg^{-1} Au-NPs (60.3 ng g^{-1} and 72.2 ng g^{-1} , respectively) (Balasubramanian *et al.*, 2010).

Compared to polymer conjugates discussed earlier, there is no breakdown mechanism for many metal nanoparticles, and so they remain in tissues for extended periods of time. The tissue retention of metal nanoparticles is a particular concern in the light of the ability of metal nanoparticles to create oxidative stress through transition metals on the surface of the nanoparticle or the release of metal ions from the nanoparticle. Zinc oxide nanoparticles (ZnO-NPs), for example, are commonly used in sunscreens and cosmetic agents. Oral exposure of mice to ZnO-NPs resulted in oxidative stress induction in the liver and kidney (Sharma *et al.*, 2012). Such observations raise the concern that metal nanoparticles may contribute to the induction of a variety of oxidative stress-related pathologies (Sharma *et al.*, 2012).

3 ROUTES OF EXPOSURE

Although the composition of the nanomaterial is important for the adverse events that may occur

following exposure, the route through which an organism is exposed can play a pivotal role in the toxicity produced. The route of entry usually involves a specific organ being exposed to a sudden high concentration of nanoconstructs.

First-pass organs such as those comprising the gastrointestinal (GI), integumentary, pulmonary, and vascular system can show independent and unique toxicity profiles. Following are some examples of how the route of exposure can be an important determinant of nanoconstruct safety.

3.1 Gastrointestinal Exposure

The GI tract is one of the largest immunological organs of the body, containing more lymphocytes and plasma cells than the spleen, bone marrow, and lymph nodes (Holmgren *et al.*, 1992). Exposure of the GI to nanoparticles can be due to the presence of nanoparticles in the environment or intentional exposure in the form of oral nanomedicines. In addition, nanoparticles cleared from the respiratory tract via the mucociliary escalator can subsequently be ingested into the GI tract.

Copper nanoparticles have a wide array of applications as antibacterial coatings and lubricants (Cioffi *et al.*, 2005; Liu *et al.*, 2004). A study by Chen *et al.* (2006) has emphasized that the importance of particle size for the toxicity induced through nanoparticle exposure to the GI tract. They compared the effect of gastrointestinal exposure to $17 \mu\text{m}$ and 23.5 nm sized copper particles. It was observed that mice exposed to the nano-sized particles experienced loss of appetite, diarrhea, and vomiting, whereas exposure to micro-sized particles was relatively safe (Chen *et al.*, 2006). Another example of specific GI toxicity on exposure to nanoparticles was documented with the use of ZnO-NPs. ZnO-NPs are being investigated for applications in food preservation because of their antimicrobial action (Espitia *et al.*, 2012). This application can result in oral exposure to ZnO-NPs. Mice treated with 20 nm and 120 nm ZnO-NPs through oral gavage showed a dose-dependent toxicity indicating pathological damage to the GI tract manifested by inflammation in the lamina propria and diarrhea (Wang *et al.*, 2008).

Various carriers are also being developed for oral nanomedicine such as silica nanoparticles for inflammatory bowel disease (Lamprecht *et al.*,

2001) and chitosan nanoparticles for oral insulin delivery (Makhlof, Tozuka, and Takeuchi, 2011). Though GI exposure to nanoparticles has been found to pose specific toxicity, data related to safety of this route of exposure is still sparse and warrants more studies.

3.2 Dermal Exposure

The skin is the largest and the most exposed surface of the body that increases the likelihood of being exposed to nanomaterials. Dermal exposure can occur through the application of nanomaterials onto the skin, such as through the use of lotions or sunscreens containing nanoparticles (Basavaraj, 2012), through the use of bandages incorporated with silver nanoparticles, (Ag-NPs) to prevent infection (Tredget *et al.*, 1998), or by the deposition of nanoparticles onto the skin through environmental exposure.

A 28-day subchronic toxicity test showed that ZnO-NPs (75 mg kg^{-1}) applied to the skin of Sprague–Dawley rats had the ability to decrease the collagen content of the skin and tail 63.9 and 53.5%, respectively (Surekha *et al.*, 2011). Surekha *et al.* hypothesized that the loss of collagen in the skin may have been a consequence of oxidative stress induced by the ZnO-NPs, which is a common side effect of metal nanoparticle exposure and that the differential effect on the skin and tail may have been a result of differential penetration. In contrast, application of 2000 mg kg^{-1} of micro-sized ZnO particles resulted in collagen content decreasing only 10.8% in the skin and 7.94% in the tail (Surekha *et al.*, 2011). The observation of collagen loss following repeated exposure to ZnO-NPs is a matter of concern considering their use in sunscreens and cosmetics that are often applied daily for extended periods of time. Ag-NPs applied dermally were found to show no acute dermal toxicity in rats and rabbits up to 2000 mg kg^{-1} of body weight (Jain *et al.*, 2009; Kim *et al.*, 2012) making their utilization as topical antibacterial agents a promising option. However, Ag-NPs were found to exhibit slight skin-sensitizing effects in a skin sensitization test utilizing guinea pigs (Kim *et al.*, 2012).

Although some lipid-soluble nanoparticles can penetrate the skin through the pathways between the stratum corneum cells, through transcellular pathways or through the hair follicle and sweat

ducts (Pflucker *et al.*, 2001), the rate and quantity of absorption depend on the nature of the nanomaterial. The recent progress in nanomedicine design has allowed novel approaches in the development of lipid carriers for dermal penetration, making them suitable candidates for treatment of eczema (Schäfer-Korting, Mehnert, and Korting, 2007) as topical treatment for this skin disease may lower the risk of systemic side effects.

However, many nanoparticles are unable to penetrate the intact skin and so investigation of toxicity in the presence of disruptions of the skin such as wounds, scrapes, or dermatitis is necessary.

3.3 Respiratory Tract Exposures

Respiratory exposure to nanoparticles can occur in a variety of circumstances such as occupational, environmental, or cosmetic. A particularly glaring example of exposure is the collapse of the World Trade Centre, wherein thousands of local residents, local employees, and emergency response personnel were exposed to a wide variety of nano-sized materials from combustion products to particles of concrete and glass (Lioy, Pellizzari, and Prezant, 2006).

Manganese oxide (MnO_2) ultrafine particles constitute a major part of welding fumes, rendering them an occupational hazard for welders (Norihiko and Yasushi, 2006). Rats, after 11 days of exposure to MnO_2 (30 nm), showed a 30-fold increase of TNF- α , macrophage inflammatory protein-2, and glial fibrillary acidic protein in the olfactory bulb (Elder *et al.*, 2006) indicating the ability of these particles to translocate through the olfactory neuronal pathway.

CNTs are used widely in textiles because of their tensile strength (Endo *et al.*, 2004); however, concerns have risen over the ability of CNTs to induce inflammation and granulomatous lesions when injected into the peritoneum of mice, in a manner mimicking the granuloma induction by asbestos (Poland *et al.*, 2008). Although this evidence was criticized because of the use of the peritoneal cavity as a model for the pleural cavity, exposure of the respiratory system via pharyngeal aspiration of SWCNTs has also showed acute inflammation, early formation of granulomas, and progressive fibrosis (Shvedova *et al.*, 2005). Furthermore, subchronic, nose-only exposure of Wistar rats to MWCNTs also

resulted in lesions at in the upper respiratory tract, increased interstitial collagen, and inflammation (Pauluhn, 2010). This particular adverse effect highlights the importance of examining toxicity via all potential routes of exposure as intravenous exposure of mice to CNTs does not induce granulomas (Yang *et al.*, 2008).

Respiratory administration of nanomedicine is also being pursued for local treatment of conditions such as asthma (Ungaro *et al.*, 2012) or to achieve entry into the systemic circulation via the large surface area of the alveolar region, the rich vascularization, and the thickness of the epithelial barrier. Liposomes are being investigated for controlled delivery to the lung as these constructs can be formed from compounds naturally found in the lungs and be used to encapsulate compounds such as antibiotics for tuberculosis treatment (Justo and Moraes, 2003). However, care must be taken with the utilization of these constructs as cationic liposomes have shown the ability to induce inflammation following intratracheal instillation (Dokka *et al.*, 2000).

3.4 Intravenous Route Exposures

Intravenous administration of nanomedicines allows 100% bioavailability and represents a unique and important exposure route for nanomedicines being used for therapy, imaging, or diagnosis. This route of administration may result in unique nanomedicine toxicity via effects on the components of the cardiovascular system such as the thrombotic system, complement system, red blood corpuscles, and the endothelial cells lining the vascular tree.

Activation of the thrombotic cascade is a potentially life-threatening adverse event and has been observed following the intravenous administration of a variety of nanoconstructs (Yu *et al.*, 2012; Geys *et al.*, 2008). The activation of this cascade may be modulated via the modification of the nanoconstruct or by appropriate management of patient parameters. For example, Geys *et al.* (2008) observed that when carboxyl or amine quantum dots composed of cadmium, selenium, and zinc (CdSe/ZnS) were administered to mice, both constructs induced pulmonary thrombosis; however, carboxyl quantum dots produced significantly more thrombi than amine quantum dots (Geys *et al.*, 2008). Preadministration of heparin to mice abolished the formation of

thrombi, indicating that the thrombotic effect of these quantum dots was dependent on the activation of the secondary coagulation cascade and may be appropriately managed for patient safety (Geys *et al.*, 2008).

Although Geys *et al.* found carboxyl quantum dots to be most problematic, this effect cannot be attributed to the charge alone. Greish *et al.* (2011) have shown that amine-terminated dendrimers elicited the formation of thrombi, whereas carboxyl-terminated dendrimers did not. Subsequent research has indicated that the thrombotic action of the amine-terminated dendrimers was related to direct interaction with fibrinogen in a thrombin-independent manner (Jones *et al.*, 2012). The inconsistency between the thrombotic effect of charge when examining quantum dots and dendrimers emphasizes the importance of understanding the multiple characteristic of nanomedicines that modulate the *in vivo* response.

As outlined earlier, the other nanoparticles administered intravenously, such as the FDA-approved liposome Doxil, which encapsulates doxorubicin, elicits a rather unique adverse effect known as *C activation-related pseudoallergy*. This adverse event can result in benign symptoms such as flushing or in life-threatening conditions such as arrhythmias and ventricular fibrillation (Szebeni *et al.*, 2006). Pseudoallergy, which is further discussed in the special tests section, subsides with repeated administration but is still a clinical concern as it is life threatening in 0.9% of patients (Szebeni, 2005).

4 EVALUATION OF MATERIAL TOXICITY

4.1 Toxicity of Nanomaterials

As outlined earlier, the physicochemical characteristics of nanomaterials greatly impact their toxicity. *In vivo* nanotoxicity may be tested following acute, subchronic, or chronic exposure. Acute exposure occurs following a single administration of the substance, whereas subchronic exposure involves repeated administration for up to 10% of the animals' life span. Finally, chronic testing is achieved over 50 to 100% of the animals' life span and is the gold standard of toxicity testing. Chronic exposure allows the detection of adverse effects with delayed onset or that are related to aging. The

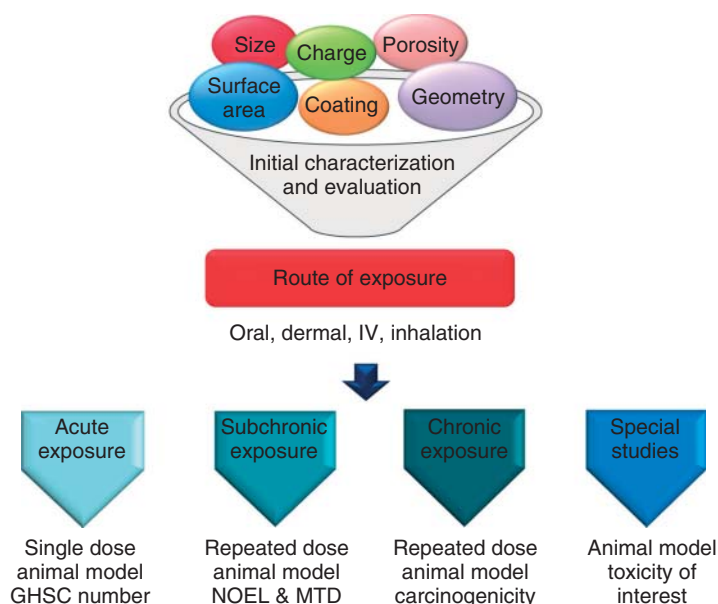


Figure 1. *In vivo* testing of nanotoxicity. The figure outlines the initial characterization and evaluation of nanomaterials that must be made before *in vivo* testing. The figure also shows the range of *in vivo* tests that should be employed in order to adequately assess the toxicity of nanoconstructs.

important parameters of *in vivo* testing are outlined in Figure 1. In the next section, we will provide methodologies approved by the Organization for Economic Cooperation and Development (OECD). The OECD represents 34 countries and provides approved toxicity tests to allow the ease of comparison of data between independent groups and clear guidance regarding protocols that should be employed. This effort represents the latest development toward unified nanotoxicology testing. We discuss the relevance, appropriate application, and necessity of these studies while highlighting several adverse effects that are of particular concern following nanoparticle and nanomedicine exposure.

4.2 Acute and Chronic Testing of Toxicity

4.2.1 Acute Toxicity

Acute Oral Toxicity

In the acute oral toxicity test, groups of animals of single sex are given stepwise doses of 5, 50, 300, and 2000 mg kg⁻¹, although higher or lower fixed doses may be administered depending on the toxicity or mortality profile. Rodents are usually the preferred animal model for testing because of

ease of use and the thorough characterization of the various strains. Females are the preferred sex if there is no property of the compound expected to produce gender-dependent toxicity as, when differences in sensitivity are observed between the sexes, females are generally more sensitive (Lipnick *et al.*, 1995). Females should be nulliparous and nonpregnant unless teratogenicity is an end point of interest and treatment should begin at 8–12 weeks of age. The administration route may be through food or by oral gavage. Oral gavage is preferred because of the ability to control the dose precisely. If administration of the nanoconstruct is in food, as is expected for food packaging or preservation methods, fasting is not appropriate. However, if administration is via oral gavage, the animals should be fasted overnight. This may be particularly important for nanomedicine administration if the construct is sensitive to degradation by bile or digestive enzymes.

A single animal should be administered a single dose with a delay period of at least 24 h between dosing of the next animal and observation should be carried out for at least 14 days. A total of five animals should be used for each dose and a period of 3–4 days should be allowed between the dose

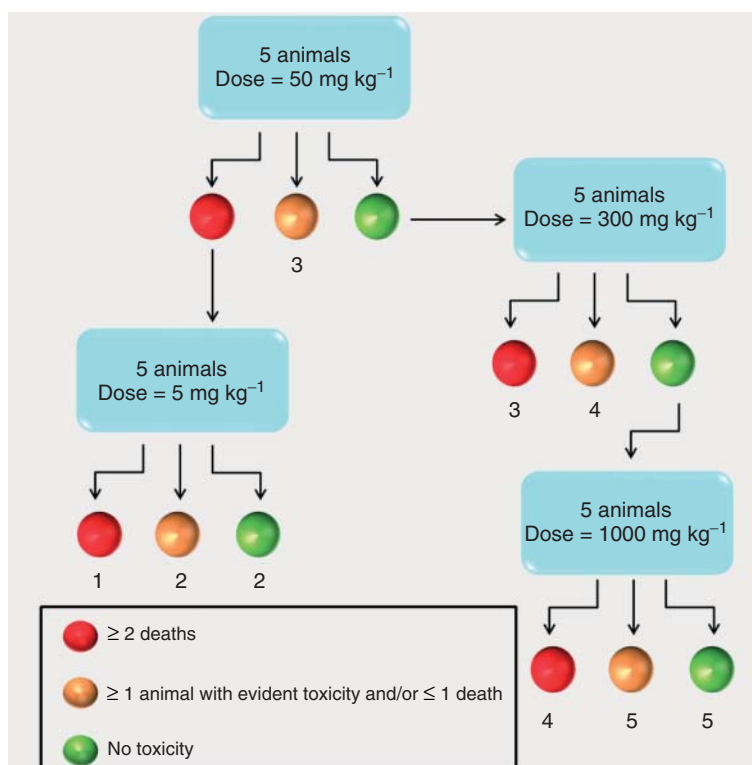


Figure 2. Oral fixed dose procedure and substance classification. The starting dose is determined by a sighting study and the dose is adjusted according to the effects observed. The number given denotes the Globally Harmonized system of Classification number, which is used to give consistent classification of compounds toxicity (OECD). In the classification, 1 represents the most toxic compounds, whereas 5 denotes the least toxic/unclassified compounds (please refer to Acute Oral Toxicity).

escalations in order to observe delayed toxicity. Animals must be examined within the first half hour following dosing and daily thereafter for a total of 14 days unless found dead or sacrifice is necessary. The toxicity observed is used to classify the compounds toxicity according to the globally harmonized system of classification as is outlined in Figure 2. Observations outlined in Table 1 should be undertaken. Following sacrifice, gross necropsy should be pursued and the organs outlined in Table 1 should be examined to identify gross pathological changes. This test has been previously used to examine the toxicity of titanium oxide (TiO₂) (Wang *et al.*, 2007) and ZnO-NPs (Wang *et al.*, 2006).

Acute Dermal Toxicity

The procedure for acute dermal toxicity suggests the use of rodents, rabbits, or guinea pigs of a single sex, preferentially female as is specified for acute

oral toxicity. Approximately 10% of the body fur must be removed from the dorsal section of the trunk, taking care to avoid abrading the skin. This is particularly important for nanoconstructs as they would not normally pass through intact skin and show higher penetration following dermal abrasion (Gopee *et al.*, 2009). However, if the nanoconstruct in question is being developed for use following skin damage, that is, creams to assist wound healing, then skin abrasion may be preferable. The test should use at least five individuals in each treatment group and at least three different doses of the test substance for estimation of the LD₅₀. The dose should be applied to the hairless skin in a thin, uniform layer, and held in place using a porous gauze dressing and tape for a total of 24 h before removing the substance with a suitable solvent. The animals should be monitored closely within the first 24 h and at least one observation made every 24 h for a total of 14 days, paying attention to the observations

Table 1. Toxicology observations.

Observations	Body weight
	Gross necropsy
	Skin
	Fur
	Eyes
	Mucous membranes
	Respiratory system
	Nervous system
	Circulatory system
	Motor activity/behavioral patterns
	• Tremors • Convulsions • Salivation • Diarrhea • Lethargy • Sleep • Coma
	Pathological lesions
Hematology	Food and water consumption
	Total and differential leukocyte counts
	Erythrocyte count
	Platelet count
	Reticulocytes
	Hemoglobin concentration
	Hematocrit
	Mean corpuscular volume
	Mean corpuscular hemoglobin
	Mean corpuscular hemoglobin concentration
	Prothrombin time
	Clotting time
Clinical biochemistry	Activated partial thromboplastin time.
	Glucose
	Urea
	Creatine
	Total protein
	Albumin
	Calcium
	Sodium
	Potassium
	Phosphorus
	Chloride
	Globulin
	Total cholesterol
	Triglycerides
	At least two appropriate tests for hepatocellular evaluation
	• Alanine aminotransferase • Aspartate aminotransferase • Glutamate dehydrogenase • Total bile acids
	At least two appropriate tests for hepatobiliary evaluation
	• Alkaline phosphatase • Gamma glutamyl transferase • Total bilirubin • Total bile acids

*(continued)***Table 1.** (Continued)

Urinalysis	Appearance
	Volume
	Osmolarity/specific gravity
	pH
	Total protein
	Glucose
	Ketones
	Bilirubin
	Occult blood
	Adrenals
Gross necropsy organs of interest	Aorta
	Bone marrow
	Brain
	Caecum
	Colon
	Duodenum
	Femur and stifle joint
	Gallbladder
	Heart
	Ileum
	Jejunum
	Kidneys
	Larynx
	Liver
	Lung
	Lymph nodes
	Mammary gland (female)
	Muscle
	Nasopharyngeal tissues
	Oesophagus
	Ovaries
	Pancreas
	Parathyroids
	Peripheral nerve
	Pituitary
	Prostate
	Rectum
	Salivary glands
	Seminal vesicles
	Skin
	Spinal cord
	Spleen
	Sternum
	Stomach
	Testes
	Thymus
	Thyroid
	Trachea
	Urinary bladder
	Uterus
	All gross lesions and masses

described in Table 1. This test has previously been used to assess the safety of Ag-NPs (Jain *et al.*, 2009; Kim *et al.*, 2012).

Acute Inhalation Toxicity

Rats are the preferred species and should be exposed from 8 to 12 weeks. A typical exposure should last for 4 h and should ideally be through the nose, as methods such as cage exposure present the risk of the animal ingesting the substance while grooming. If a susceptible sex is known, five members of that sex should be used; however, if no susceptibility is known, five animals of each sex should be used. At least three concentrations should be used in order to show a dose–response relationship and the doses should be staggered in order to confirm that the animals administered with the previous dose have survived. Observation of the treated animals should be made at least twice on the day of exposure, more frequently if signs of distress are noted, and at least once every day after, for a total of 14 days. If there is an indication that the toxicity of the compound may be delayed, then the observation period may be extended.

The nanoparticles must be suspended in air at an appropriate size and concentration that may require a vehicle, preferably water. It is recommended that the nominal concentration of the compound (initial mass of compound before suspension/total volume of air) and the actual concentration (mass of compound suspended/total volume of air) of the compound, be assessed in order to determine if the method used to produce an air suspension is successful. Only actual concentration should be used for statistical purposes. The mean concentration in individual inhalation chambers, monitored at least twice in a 4 h exposure, should not vary more than 10 or 20% from the mean for gasses and liquids/solids, respectively. At least twice during any 4 h exposure, the particle size distribution should be assessed using a primary instrument, such as a cascade impactor or aerodynamic particle sizer, and a secondary device such as a gravimetric filter or impinge/gas bubbler to ensure accuracy of the primary test instrument. Particle size determination is particularly important when assessing the toxic potential of nanoconstructs as factors such as surface area have been shown to be a determining factor for the generation of reactive oxygen species (Singh *et al.*, 2007). The observations outlined in Table 1 should be assessed and necropsy should

be performed on all animals and gross lesions assessed with particular attention paid to the respiratory tract. Parameters such as lung lavage and perhaps penetration of the nanoconstruct through respiratory tissue may be required for specific nanoconstructs, especially regarding CNTs where penetration of the pleura is an important factor for the potential to induce mesothelioma. This procedure has previously been used for evaluation of cerium oxide nanoparticles (Srinivas *et al.*, 2011), 1,6-Hexamethylene Diisocyanate Homopolymer (Pauluhn, 2000), and Ag-NPs (Sung *et al.*, 2011b).

4.2.2 Subchronic Toxicity

Repeated Dose Oral Toxicity Study—90 day Study

The test is performed over 10% of the animal's life span and suggests the use of rodents with a body weight of $\pm 20\%$ of mean weight. The test should employ 10 animals of each sex at each dose level. It is also recommended that there be one control group and at least three dose levels, to identify the no observable effect limit (NOEL) with the lowest dose and to identify the MTD with the highest dose. If administered via oral gavage, exposure should commence before 9 weeks of age and animals should be treated 7 days per week for 90 days. Animals should be observed at least twice daily in order to assess clinical well-being and detect signs of morbidity or mortality. The observations outlined in Table 1 should be considered with the addition of changes in gait, posture, tonic/clonic movements, stereotypy, or other abnormal behavior such as self-mutilation. Sensory reactions to stimuli (e.g., auditory and visual) should be examined toward the end of the exposure period, and consumption of food and water should be monitored. The subchronic oral toxicity methods have previously been employed for testing of Ag-NPs (Kim *et al.*, 2010) and ZnO-NOs (Seok *et al.*, 2013).

Subchronic Dermal Toxicity—90 day Study

Animals employed for dermal toxicity studies should be rats, rabbits, or guinea pigs and at least 10% of the body fur should be removed from the dorsal area of the trunk, ensuring care is taken to avoid abrading the skin. At least 10 animals of each gender should be used in each dose level, and females should be nulliparous and nonpregnant unless teratogenicity is an end point of interest. A control and at least three dose levels should be

employed, with the aim of identifying the NOEL and MTD. Animals should be exposed to the substance by application of a uniform film onto the hairless area for 6 h per day, 7 days per week for a total of 90 days. The substance should be held in place by a porous gauze and tape to ensure it is not ingested. Observations as outlined in Table 1 should be undertaken along with weekly observations of food and water consumption, ophthalmological health, and weight. Hematological and clinical biochemistry analyses should be carried out as outlined in Table 1.

Subchronic Inhalation Toxicity—90 day Study

Animals are exposed for 90 days via the inhalation route to the substance of interest and Rodents (7–9 weeks) are the preferred species and at least 10 animals of each sex should be exposed in each test groups for 6 h per day, 5–7 days per weeks for a total of 90 days. There should ideally be three concentration levels employed as well as a vehicle or negative (air) control. The concentrations should be chosen with an aim to enable statistical evaluation of a concentration response to identify the NOEL and MTD.

Particle size distribution, nominal concentration, and actual concentration should be assessed as outlined in the acute study. Observations as outlined in Table 1 should be pursued regularly with the addition of rectal temperature to provide information on the possible development on reflex bradypnea or hypo/hyperthermia related to treatment or confinement in the nose only exposure apparatus. The assessment of the nanomaterials' kinetics, retention, and the subsequent effect on lung function is particularly important in the exposure of nanomaterials because of the high durability of some constructs. Urinalysis is not routinely required but may be necessary for some specific nanoconstructs. Bronchioalveolar lavage is recommended if the nanoparticle is expected to be retained in the lower respiratory tract and in order to quantify parameters such as alveolitis, pulmonary inflammation, phospholipidosis, fibrosis, proinflammatory cytokines/chemokines, or lysosomal injury. Gross necropsy should be performed assessing all of the organs outlined in Table 1. Particular attention should be paid to the respiratory tissues including unlavaged lungs, instilled with a suitable fixative to maintain structure. It is recommended that nasopharyngeal tissue from at least four different

areas including the nasopharyngeal duct, lymphatic tissue, respiratory, and olfactory epithelium be examined. The standardized subchronic inhalation toxicity test has previously been utilized for investigation of toxic effects of Ag-NPs (Kim *et al.*, 2011), Au-NPs (Sung *et al.*, 2011a), and CNTs (Ma-Hock *et al.*, 2009).

Chronic Toxicity

The standardized chronic toxicity test (OECD) allows for the testing of chronic exposure via the oral, dermal, or inhalation route. This test combines toxicity studies with carcinogenicity studies. This test is the recommended protocol as it allows reduction of time, cost, and animal numbers. Rodents are the preferred animal model because of their short life span, susceptibility to tumor induction, and the widespread use of these models within pharmacological and toxicological studies. Rats are preferred over mice as the use of mice can require greater numbers of animals in order to collect sufficient amounts of blood to conduct the hematological studies.

To examine chronic toxicity and carcinogenicity, the substance is administered daily to several groups of test animals, one dose level per group and at least three dose levels. This test is normally performed over 12 months or less as exceeding this duration may confound with the effects of aging. The doses should be chosen in order to establish a dose response and a NOEL. At least 50 animals of each sex should be used in each treatment group although larger numbers may be necessary to detect differences in lower dose groups. Exposure should be performed as follows:

Dermal—6 h per day 7 days per week

Oral—oral gavage or in food and
water 7 days per week

Inhalation—6 h per day 7 days per week

The measures of toxicity employed should be thorough, broad, and informed by previous acute and subchronic studies. Observations of physical health, movement, food and water consumption, behavior, ophthalmological examination, neurological health, and immunotoxicity if previously indicated in acute or subchronic studies. Special care should be taken to conduct thorough hematological and urinalysis studies (Table 1).

Carcinogenicity testing should involve treatment for 24 months, the typical life span of a rat. Gross

necropsy should be performed but organ weight, due to geriatric changes, may not be informative and so is not required. Collection and examination of target organs, as outlined in Table 1, should be assessed. In addition, tumor development, time of onset, location, dimension, appearance, and progression should be recorded. Morbidity and mortality should be assessed twice daily and signs of toxicity should be checked for once daily.

Special Tests

Although the standardized testing procedures are thorough, they may not address particular concerns that may be unique to a nanoconstruct. For this reason, we have outlined particular concerns regarding exposure to nanoconstructs and the protocols that could be employed in order to appropriately test these potential adverse events.

Hepatotoxicity

Nanomedicine is used primarily to exploit the enhanced vascular permeability of tumor tissue to increase drug delivery to the tumor (Taurin, Nehoff, and Greish, 2012). However, other body tissues such as the liver, which has large vascular fenestrations regardless of disease state, will also experience increased nanomedicine concentration (Taurin, Nehoff, and Greish, 2012). The quantity of intravenously injected nanomedicine being deposited in the liver can exceed 50% of the administered dose (Perrault *et al.*, 2009). Owing to these observations, the measurement of hepatotoxicity is crucial for preclinical testing of any nanoconstructs, intended for medical use.

Measurement of hepatotoxicity can be determined indirectly, through the measurement of enzyme levels in the blood (Table 1), and directly through histological examination. Regular estimation of the health of the liver may be assessed in live animals by measuring the levels of alanine aminotransferase and aspartate aminotransferase in the blood, which are found in high concentrations in hepatic cells and are released following trauma. Elevated bile salts or bilirubin in the serum may indicate impaired uptake or excretion such as observed following CNT administration (Ji *et al.*, 2009).

Previous research has shown that treatment with various nanoconstructs such as silica and Ag-NPs (Kim *et al.*, 2008; Chen, Xue, and Sun, 2013) can result in inflammation which is commonly observed with hepatic damage. Hepatic inflammation may be assessed indirectly by evaluating

the level of inflammatory cytokines in the blood such as interleukin-1 β and TNF- α (Xiao *et al.*, 2011; Chen, Xue, and Sun, 2013). This method allows ongoing monitoring of the inflammation to assess parameters such as duration and recovery. At desired end points, liver samples may be assessed for histopathological indications of inflammation by examination of inflammatory cell infiltration and biomarkers of oxidative stress such as 8-OHdG (Xiao *et al.*, 2011; Chen, Xue, and Sun, 2013).

Histological examination of fixed slices of hepatic tissue allows identification of other markers of hepatotoxicity such as fibrosis. Hematoxylin and eosin (H & E) staining allows identification of areas of necrosis, fibrosis, nuclear polymorphism, and excess fat deposition, whereas apoptosis may be assessed via utilization of a terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay. The classification of hepatic damage may be ranked according to the Roenigk Classification (Berends *et al.*, 2007).

Mesothelioma

Exposure to asbestos has been known to cause the formation of a rare form of cancer known as *malignant mesothelioma*, which most commonly occurs in the pleural cavity of the lungs but may also occur in the peritoneum and pericardium (Robinson, Musk, and Lake, 2005). This phenomenon was determined to be a consequence of the long, filamentous, high aspect ratio structure of the asbestos rather than the chemical composition. CNTs display a remarkable structural similarity to asbestos raising concern that exposure to CNTs may result in a similar side effect profile. The use of CNTs, as asbestos was in the 1950s, is now widespread with the market value of CNTs estimated to reach US \$7.72 billion by 2015 (Venkata Ramana, Yu, and Seshaiiah, 2013).

The risk of mesothelioma following exposure to CNTs can be evaluated in short-term or long-term studies. Short-term studies (1 month or less) are restricted to detection of precursors of mesothelioma, such as markers of inflammation and the formation granulomas, which are groupings of frustrated macrophages that have failed to eliminate the persistent fragment. The long-term studies (up to 2 years) are able to detect the formation of mesothelioma. The model most often used is a peritoneal model, wherein CNTs are injected into the peritoneal cavity of rodents (Figure 3). The peritoneal cavity in rodents, between the parietal

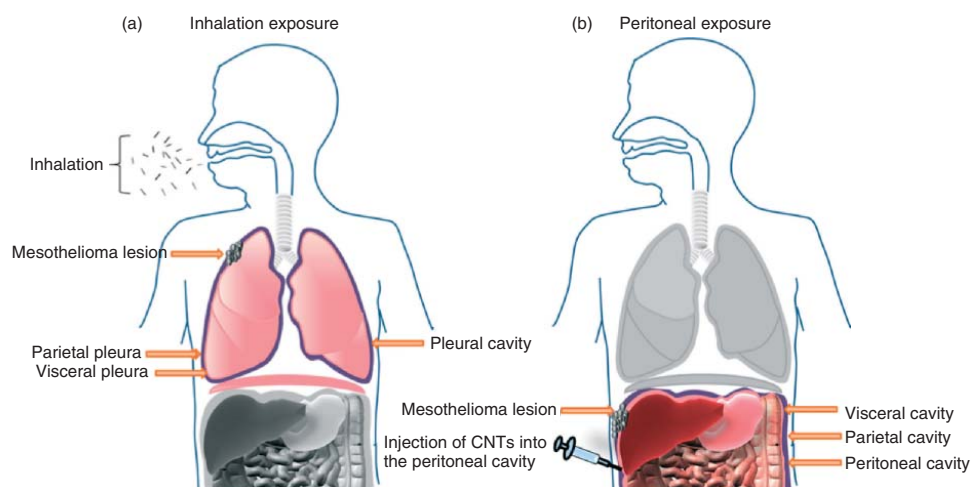


Figure 3. Inhalation and peritoneal models of mesothelioma. (a) Inhalation exposure, which better represents the environmental exposure, is used for testing development of granuloma/mesothelioma in the pleural cavity. (b) Peritoneal exposure, wherein the peritoneal cavity is used to model the pleural cavity.

and the visceral peritonea in the abdomen, is used as a model for the pleural cavity, which is found between the visceral and the parietal pleurae of the lungs. The parietal cavity has previously been used as a model for the pleural cavity to establish induction of inflammation and mesothelioma following peritoneal exposure to asbestos because of the ease of administration of uniform quantities of CNTs and the similarity of anatomy and physiology (Donaldson *et al.*, 1989; Davis, 1976; Donaldson *et al.*, 2010). Following exposure and sacrifice, the peritoneal cavity may be lavaged and the quantity of inflammatory proteins and leukocytes present in the peritoneal space quantified. The internal structures of interest such as the diaphragm may be removed, fixed, and stained in order to identify granulomatous lesions or mesothelioma lesions (Poland *et al.*, 2008; Takagi *et al.*, 2008).

The inhalation model of CNT exposure is the best model available. This model allows exposure to CNTs through the same route as human exposure would occur, repeated administration over months to years as would occur in exposure in a workplace environment and evaluation of the kinetics of the CNT within the respiratory tract. A 13 week subchronic inhalation model has been outlined by the OECD and has been previously utilized for the examination of CNTs (Pauluhn, 2010). Briefly, male and female rats are exposed to micronized CNTs in inhalation chambers for 6 h/day, 5 days/week for 13 weeks. Following exposure, the rats are sacrificed. Bronchial lavage then can be used to determine the number of cells present in the lavage fluid, the type of cells present, cell morphology, and the presence of other proteins of interest such as soluble collagen. These parameters can give information regarding the level of inflammation and extent of fibrosis that are often associated with the formation of mesothelioma. The use of the inhalation model also allows the total amount of CNTs deposited in the lungs to be assessed for parameters of interest such as agglomeration, retention time, and distribution. The tissue of unlavaged lungs, trachea, and nasal passages may be fixed for histopathological analysis to identify fibrosis, oxidation, inflammation, and granulomatous or mesothelioma lesions.

Thrombosis

Various nanoconstructs such as CNTs, quantum dots, and MSNs have been observed to cause

obstruction of blood vessels, either through the aggregation of nanomedicine inside blood vessels or by activation of the primary or secondary clotting cascade (Cheng *et al.*, 2012; Geys *et al.*, 2008; Radomski *et al.*, 2005; Bihari *et al.*, 2010). The rodent is the most commonly used model for examination of nanoconstruct-induced thrombosis. Most studies will use only male mice because of estrogen enhancing the capacity of blood to clot (Jick *et al.*, 1995) serving as a potentially confounding variable.

Thrombosis can occur as a result of the primary coagulation cascade, which is activated when platelets come into contact with the exposed collagen of damaged blood vessels, or the secondary coagulation cascade, wherein fibrin is formed through activation of the intrinsic or extrinsic cascade. Whether or not the thrombosis is primarily a consequence of the primary or the secondary cascade can be determined by pretreating animals with heparin, which will prevent fibrin cross-linking in response to secondary cascade activation (Geys *et al.*, 2008). Analysis of the relative amounts of activated platelets may be assessed via analysis of P-selectin in blood samples collected in a heparinized tube from treated animals before sacrifice. P-selectin is a protein found in α -granules in inactivated platelets but expressed on the platelet surface following activation (Geys *et al.*, 2008). This is an important distinction because activation of the secondary coagulation cascade may be a consequence of interactions with serum proteins that may be reduced via modification of charge or surface functionalization (Geys *et al.*, 2008).

If the parameter of interest is the duration of clot persistence or the impact of the clot on the blood flow to a particular organ, the blood flow can be examined in real time if necessary. The use of ultrasound is preferred because of its versatility, ease of use, and the ability to quantify blood flow.

The incidence of thrombosis can also be examined histologically following sacrifice. Following sacrifice, organs such as the liver, lungs, brain, heart, kidneys, and spleen should be examined. Although the clot itself cannot be reliably identified by fixing, slicing, and staining the tissue with an H & E stain, the effects of the clot can be identified in the form of necrotic regions and/or fibrosis.

Pseudoallergy

The complement system is a group of serum proteins that is part of the innate immune system that enhances the ability of phagocytic cells to clear pathogens. Complement activation is a common occurrence following administration of nanomedicine, particularly liposomes. This reaction is termed a *C activation-related pseudoallergy*, which is life threatening in 0.9% of patients (Szebeni, 2001; Szebeni, 2005; Johansson *et al.*, 2004). Symptoms occur immediately following administration and result in various forms of anaphylaxis such as flushing, respiratory, and circulatory disturbances in up to 45% of patients (Szebeni, 2005; Szebeni *et al.*, 2006).

In vivo analysis of C activation-related pseudoallergy can be conducted in dog and pig models, whereas rodents are deemed unsuitable because of reduced hypotensive responses to liposomes. The pig model is the most sensitive to the effects of phospholipid administration in the form of liposomes and shows both cardiovascular and cutaneous responses to similar quantities of phospholipid as in humans (Szebeni *et al.*, 2007).

The measurement of cardiac parameters in pigs is undertaken in anesthetized animals wherein a catheter in the pulmonary artery will record pulmonary artery pressure, central venous pressure, and cardiac output. A catheter in the proximal aorta may be used to measure systemic arterial pressure, and a catheter in the left ventricle will provide measurements of left ventricular end-diastolic pressure (Szebeni *et al.*, 1999; McLoughlin *et al.*, 1996). An electrocardiogram can be effectively employed to identify arrhythmias. Blood samples may be withdrawn to assess parameters related to the pseudoallergy response such as vasoconstrictors and blood hemolytic complement (Szebeni *et al.*, 1999). Administration of inhibitors of activation or production of components suspected to play a causal role in hemodynamic responses to liposome administration may lend further evidence of the mechanisms involved.

Pulmonary Fibrosis

Exposure of the lung to nanomaterials may be the result of therapeutic treatments developed for treatment of conditions such as for asthma and cancer, or through environmental exposure (Oyarzun-Ampuero *et al.*, 2012; Zarogoulidis *et al.*, 2012).

When assessing pulmonary fibrosis, exposure may be through aerosolized nanoparticles or via intratracheal instillation. Administration should follow the expected exposure pattern as closely as possible, for example, long-term daily exposure to model environmental exposure and shorter term, intermittent exposure to model treatment of asthma attacks. Following the treatment period, animals are sacrificed in order to undergo bronchiolar lavage (BAL), to determine the number and type of cells present. BAL also allows the identification of a variety of cytokines implicated in the fibrotic process such as TGF- β , PDGF-AA, TNF- α , IL-1, IL-2, IL-4, IL-5, IL-6, IL-10, and INF- γ (Park *et al.*, 2011; Wang *et al.*, 2011).

Lung tissue is harvested, fixed, and stained for examination of fibrotic lesions and altered morphology and it is suggested that the lung tissue be homogenized for quantification of total collagen production. It is important to note that the dispersion of nanoconstructs may be a crucial factor in whether or not fibrosis is observed. In addition, exposure of these animals should mimic environmental or clinical exposure (Wang *et al.*, 2011).

5 CONCLUSION

The use of nanoconstructs is rapidly expanding and increasing the risk of human exposure and toxicity. Many factors contribute to this toxicity including the properties of nanomaterial and the route of exposure. The *in vivo* toxicity testing of nanomaterials is essential to their safe utilization, and public safety. International efforts are under way to standardize methods for nanotoxicology evaluations. In this chapter, we discussed the safety concerns related to the major groups of nanoconstructs and guidelines for measuring the *in vivo* toxicity.

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Genotoxicity of Silver Nanoparticles*

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1 INTRODUCTION

1.1 Genetic Toxicology

Genetic toxicology is the study of damage to the DNA within living cells following exposure to chemical, physical, or biological agents. It includes not only the study of small lesions at specific sites within DNA, such as DNA adducts, DNA cross-links, alkali-labile sites, and point mutations, but also the examination of chromosomal changes, including aneuploidy (abnormal number of chromosomes), clastogenicity (chromosome fragmentation), and structural chromosomal rearrangements. Changes in sequence and normal structure of DNA can lead to a number of health problems, including cancer. For risk assessment of new substances or existing products, it is important to elucidate whether or not a compound is genotoxic. A battery of *in vitro* and *in vivo* genetic toxicology tests covering different genotoxicity end points such as the Comet, micronucleus, and mouse lymphoma assays are used for predicting the genotoxicity and carcinogenicity of tested compounds.

1.2 Genotoxic Potential of Nanomaterials

Nanomaterials are defined as materials with at least one external dimension in the size with the range

of 1–100 nm (NISOH, 2009). Nanomaterials are increasingly being incorporated into human medications and common consumer items because of their physicochemical properties. However, their unique properties, such as very small size and exceptionally large surface area to volume ratio, may confer unpredictable genotoxic properties. Hence, evaluation of their potential mutagenic and genotoxic activities is a critical step in the assessment of the safety of nanomaterials.

According to the Woodrow–Wilson database on nano-products (www.nanotechproject.org), silver nanoparticles (AgNPs) are the most commercialized nanomaterials because of their remarkably strong antimicrobial activity (Ip *et al.*, 2006; Melaiye *et al.*, 2005). AgNPs have been applied in a variety of consumer products including electronics, cosmetics, household appliances, textiles and food production, and medical products (Wijnhoven *et al.*, 2009). AgNPs have also been used to treat diseases that require constant dosing or targeting of specific cells or organs (Moghimi, Hunter, and Murray, 2001; Panyam and Labhasetwar, 2012). Recently, AgNPs have been introduced to interact with HIV-1 virus to prevent the virus from binding to the host cells (Elechiguerra *et al.*, 2005).

It has been reported that AgNPs can easily cross the nuclear membrane and interact with nuclear material, directly or indirectly (AshaRani *et al.*, 2009; Kruszewski *et al.*, 2011). These interactions may cause DNA damage or alterations and result in the formation of mutations or tumors. In this chapter, the authors review the literatures regarding

*Views presented in this chapter are those of the authors and do not necessarily reflect official positions or policies of the US Food and Drug Administration.

the genotoxicity of silver nanoparticles, elucidate potential mechanisms believed to be involved in the genotoxicity of AgNPs, and discuss possible factors that affect the genotoxicity of AgNPs.

2 GENOTOXICITY TESTINGS OF SILVER NANOPARTICLES

2.1 *In Vitro* Testing

A number of studies have shown that AgNPs induce genotoxicity in mammalian cells (Table 1). AgNPs have been reported to induce DNA breaks and micronuclei (MN), as measured with the Comet assay and MN assay, in different types of cells including mouse lymphoma cells, CHO-k1 cells, normal human lung fibroblast cells (IMR-90), human glioblastoma cells (U251), normal human bronchial epithelial (BEAS-2B) cells, human lymphoma (TK6) cells, human mesenchymal stem cells, and human hepatoma (HepG2) cells (Asha-Rani *et al.*, 2009; Grigg *et al.*, 2009; Hackenberg *et al.*, 2011; Kawata, Osawa, and Okabe, 2009; Kim *et al.*, 2011a; Kim *et al.*, 2013; Kim, Yang, and Ryu, 2010; Li *et al.*, 2011). These results suggest that AgNPs are clastogenic, and DNA-damaging agents.

AgNPs were also shown to be mutagenic in mouse lymphoma (L5178Y) cells. Treatments of mouse lymphoma cells with 5 nm uncoated AgNPs resulted in a significant and concentration-dependent induction of mutation in the *Tk* gene. Loss of heterozygosity analysis of the *Tk* mutants revealed that treatments with the AgNPs induced mainly chromosomal alterations spanning less than 34 mega base pairs on chromosome 11 (Mei *et al.*, 2012).

The genotoxicity of AgNPs in plant cells was examined as well. Different kinds of chromosomal aberrations such as chromatin bridge, chromosomal stickiness, and chromosomal breaks in root tip cells of *Allium cepa* were observed at different concentrations of AgNP-treated groups (Kumari, Mukherjee, and Chandrasekaran, 2009). In another study, AgNPs penetrated the *Vicia faba* cells and caused chromosomal aberrations and MN induction (Patlolla *et al.*, 2012).

2.2 *In Vivo* Testing

Genotoxic risks associated with AgNPs have been assessed in different *in vivo* models including drosophila, daphnia, and rodents (Table 2).

The genotoxicity of AgNPs was evaluated in drosophila using the wing somatic mutation and recombination test (Demir *et al.*, 2011). The results suggest that AgNPs are able to induce mutations, mainly via induction of somatic recombination, in drosophila. When assessing the genotoxicity with environmental models, AgNPs were found to induce DNA strand breaks in *Daphnia magna*, with a concomitant increase in mortality (Park and Choi, 2010), and in *Nereis diversicolor* (Cong *et al.*, 2011). AgNPs also caused DNA breaks and MN in roots, leaves, and/or shoots of *A. cepa* and *Nicotiana tabacum* (Ghosh *et al.*, 2012; Panda *et al.*, 2011).

The genotoxicity of AgNPs has been evaluated in rodents mainly using the *in vivo* MN, *in vivo* chromosome aberration, and the *in vivo* Comet assays. AgNPs caused a positive response in the blood cells of Wistar rats and in spleen of BALB mice evaluated with the Comet assay, and in bone marrow cells of Swiss albino male mice evaluated with chromosome aberration assay (Ghosh *et al.*, 2012; Ordzhonikidze *et al.*, 2009; Tiwari, Jin, and Behari, 2011). Long-term oral or inhalation exposures of male and female Sprague–Dawley rats to AgNPs did not cause cytotoxicity or MN frequency in bone marrow cells (Kim *et al.*, 2011b; Kim *et al.*, 2008).

3 MECHANISMS OF GENOTOXICITY OF SILVER NANOPARTICLES

Several direct and indirect mechanisms of AgNP-induced genotoxicity have been suggested in the literatures (Figure 1).

3.1 Direct Effects: DNA Binding and DNA Adducts

AgNPs have been found inside cells (Morones *et al.*, 2005) and react with phosphorus- and sulfur-containing compounds such as DNA with a high affinity (Hatchett and White, 1996). Hence, AgNPs can potentially interact with DNA and cause DNA damage, leading to mutations. One study showed that AgNPs bound DNA and compromised DNA replication fidelity (Yang *et al.*, 2009). In a previous study (Foldbjerg, Dang, and Autrup, 2011), bulky DNA adducts that could result in DNA lesions leading to mutations were found in A549 cells treated with AgNPs (Pavanello *et al.*, 2008).

Table 1. Genotoxicity of AgNPs *in vitro*.

Size and coating	Cell line	Genotoxicity assay	Assay results	References
-6–20 nm -Starch-coated	-Human lung fibroblast (IMR-90) cells -Human glioblastoma (U251) cells	-Alkaline comet assay -Cytokinesis-blocked micronucleus assay	-An increase in DNA damage in cancer cells and fibroblasts; however, extent of DNA damage was much higher in cancer cells as compared to fibroblasts -Significant numbers of micronuclei were formed in cancer cells and fibroblasts; however, more chromosomal damage was observed in cancer cells than in fibroblasts -AgNPs stimulated DNA breakage and MN formation with or without S9 mix in a dose-dependent manner -CytoB affected the genotoxicity of AgNPs	(AshaRani <i>et al.</i> , 2009)
-58.9 nm -Uncoated	-Chinese hamster ovary (CHO-k1) cells	-Alkaline comet assay -Cytokinesis-blocked micronucleus assay -Micronucleus assay without cyto B	-AgNPs stimulated DNA breakage and MN formation with or without S9 mix in a dose-dependent manner -CytoB affected the genotoxicity of AgNPs	(Kim <i>et al.</i> , 2013)
-<100 nm -PVP-coated	-Normal human bronchial epithelial (BEAS-2B) cells -Mouse lymphoma cells (L5178Y) cells	-Alkaline comet assay -Mammalian cell mutation assay in L5178Y cells	-A significantly increased tail moment (>twofold) with and without S9 in BEAS-2B cells and L5178Y cells -No significant increase in mutant frequencies in L5178Y cells	(Kim, Yang, and Ryu, 2010)
-48–55 nm -PVP coated	-Normal human bronchial epithelial (BEAS-2B) cells	-Alkaline comet assay -Cytokinesis-blocked micronucleus assay -Chromosomal aberration test -Alkaline comet assay	-Dose-dependent DNA damage occurred after both 4- and 24-h treatments -No induction of MN and CA observed	(Nymark <i>et al.</i> , 2012)
-4–7 nm -uncoated	-Human Mono Mac 6 cells -Rat alveolar macrophage	-Alkaline comet assay	-A significant increase in DNA strand breakages	(Grigg <i>et al.</i> , 2009)
-58.9 nm -Uncoated	-Normal human bronchial epithelial (BEAS-2B) cells	-Alkaline comet assay -Cytokinesis-blocked micronucleus assay -Micronucleus assay without cyto B	-A dose-dependent increase of DNA breakage -Significant MN formation in CBMN assay -Significant MN formation in MN assay	(Kim <i>et al.</i> , 2011a)
-7–10 nm -Polyethylenimine-coated	-Human Hepatoma (HepG2) cells	-Cytokinesis-blocked micronucleus assay	-An increase up to $47.9 \pm 3.2\%$ micronucleus formation in binucleated cells	(Kawata, Osawa, and Okabe, 2009)

(continued overleaf)

Table 1. (Continued)

Size and coating	Cell line	Genotoxicity assay	Assay results	References
-5 nm -Uncoated	-Human lymphoma (TK6) cells	-Micronucleus assay without cyto B in TK6 cells	-A weak positive increase in micronucleus induction in TK6 cells	(Li <i>et al.</i> , 2011)
-5 nm -Uncoated	-Mouse lymphoma (L5178Y) cells	-Mammalian cell mutation assay in L5178Y cells -Alkaline comet assay (standard and Endo III and hOGG1 enzyme modified) -Alkaline comet assay -Chromosomal aberration test -Alkaline comet assay (standard and hOGG1 enzyme modified)	-Mutagenic in L5178Y cells -No DNA damage in standard comet assay -Concentration-dependent and significant oxidative DNA damage in enzyme-modified comet assay	(Mei <i>et al.</i> , 2012)
-<50 nm -No information on coating -20 nm -Uncoated	-Human mesenchymal stem cells -HEK 293 cells	-Alkaline comet assay (standard and hOGG1 enzyme modified) -Alkaline comet assay (standard and hOGG1 enzyme modified)	-DNA damage observed in two assays -Concentration-dependent and significant oxidative DNA damages in standard and enzyme-modified comet assay	(Hackenberg <i>et al.</i> , 2011) (Hudecova <i>et al.</i> , 2012)
-20 nm -Uncoated	-Human testicular embryonal carcinoma cell (NT2) -Primary mouse testicular cells -Root tip cells of <i>A. cepa</i>	-Alkaline comet assay (standard and FPG enzyme modified) -Chromosomal aberration test	-Low levels of AgNPs-induced DNA strand breaks in NT2 cells or the primary testicular cells -No significant increase in oxidative DNA damage in both cells	(Asare <i>et al.</i> , 2012)
-<100 nm -No information on coating -60 nm -No information on coating	-Root tip cells of <i>V. faba</i>	-Chromosomal aberration test -Micronucleus assay without cyto B	-Chromosomal aberrations observed such as chromatin bridge, chromosomal stickiness, and chromosomal breaks -Chromatid aberrations observed -Statistical-significant MN induction observed	(Kumari <i>et al.</i> , 2009) (Patlolla <i>et al.</i> , 2012)

Table 2. Genotoxicity of AgNPs *in vivo*.

Size and coating	Model organism	Exposure route	Genotoxicity assay	Assay results	References
-38.67 nm -No information on coating	- <i>Drosophila melanogaster</i> : the multiple wing hairs strain and the flare-3 strain - <i>Daphnia magna</i>	-Oral	- <i>In vivo</i> wing spot assay	-Small but significant increases in the frequency of total spots	(Demir <i>et al.</i> , 2011)
-<50 nm -No information on coating	- <i>Nereis diversicolor</i>	-Dietary exposure route	- <i>In vivo</i> alkaline comet assay	-A statistical-significant increase in DNA strand breaks	(Park and Choi, 2010)
-<100 nm -PVP coated -24–55 nm -No information on coating	- <i>Allium cepa</i>	-Sediment exposure -Culture <i>A. cepa</i> in medium containing AgNPs	- <i>In vivo</i> alkaline comet assay - <i>In vivo</i> micronucleus assay - <i>In vivo</i> alkaline comet assay	-A dose-dependent increase of DNA strand breaks -Significant dose-dependent induction of cells with MN or mitotic aberrations -Significantly, dose-dependent increase in DNA damage	(Cong <i>et al.</i> , 2011) (Panda <i>et al.</i> , 2011)
-75–130 nm -No information on coating	- <i>Allium cepa</i> - <i>Nicotiana tabacum</i>	-Roots of the plants put in the water containing AgNPs -Swiss albino male mice	- <i>In vivo</i> alkaline comet assay - <i>In vivo</i> chromosome aberration assay	-In <i>A. cepa</i> roots, DNA strand breaks increased steadily up to 25 µg ml ⁻¹ followed by a gradual decrease with the increasing concentrations -In <i>A. cepa</i> shoot, the DNA strand breaks increased and reached a plateau at concentrations of 50 µg ml ⁻¹ and above -In roots and leaves of <i>N. tabacum</i> , AgNPs induced comparable significant DNA damage -Chromatid breaks in mouse bone marrow cells -An increase in DNA damage in AgNPs treatment groups. However, the comet parameter (% tail DNA) showed no further increase in DNA damage beyond the concentration of 20 mg kg ⁻¹ body weight. -Nonsignificant but a concentration-dependent increase in DNA strands breaks in spleen	(Ghosh <i>et al.</i> , 2012)
-3–15 nm -No information on coating	-BALB mice	-Intraperitoneal injection	- <i>In vivo</i> alkaline comet assay	-No micronucleus induction in bone marrow cells	(Ordzhonikidze <i>et al.</i> , 2009)
-52.7–70.9 nm -No information on coating	-Sprague–Dawley rats	-Oral	- <i>In vivo</i> micronucleus test	-No micronucleus induction in bone marrow cells	(Kim <i>et al.</i> , 2011b)

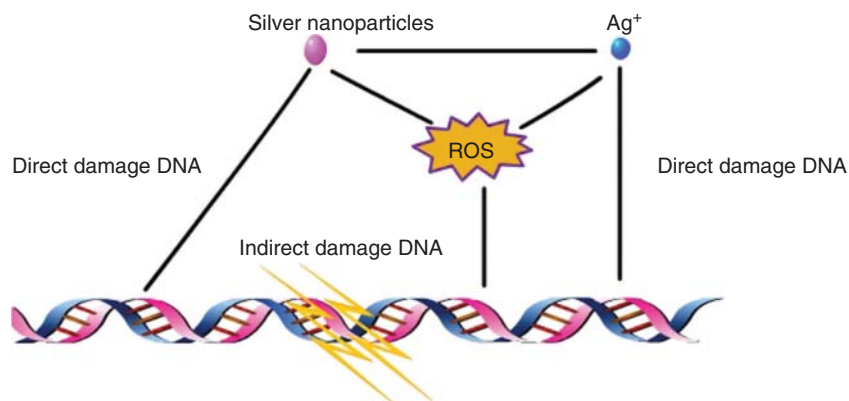


Figure 1. Schematic diagram depicting possible mechanism involved in AgNPs-induced genotoxicity.

3.2 Indirect Mechanism: Oxidative Stress

The generation of reactive oxygen species (ROS) by AgNPs is generally recognized as an indirect mechanism of AgNPs-mediated genotoxicity. It was demonstrated that AgNPs induced oxidative stress by decreasing glutathione (GSH) and superoxide dismutase (SOD) and increasing lipid peroxidation using human fibrocarcinoma HT-1080 and human skin carcinoma A431 cells (Arora *et al.*, 2008). Similar effects have also been found in human lung fibroblast cells (IMR-90) and human glioblastoma cells (U251) (AshaRani *et al.*, 2009). AgNPs can produce hydroxyl radicals in both cell-free systems (He *et al.*, 2012) and cell cultures environment (Rushton *et al.*, 2010).

High concentration of ROS eventually will lead to oxidative stress. Oxidative attack on DNA can result in mutagenic DNA adducts such as 8-hydroxyadenine and 8-hydroxyguanine. An enzyme-modified Comet assay, which by addition of enzymes that break DNA at sites with oxidative DNA adducts, showed a dose-dependent manner in oxidative DNA adducts associated with the treatment of AgNPs (Figure 2) (Mei *et al.*, 2012). The level of oxidative DNA adducts was strongly correlated with the cellular ROS levels and the addition of an antioxidant enzyme greatly reduced ROS production and the bulky DNA adducts induced by AgNPs in a human lung carcinoma cell line (A549) (Foldbjerg, Dang, and Autrup, 2011).

AgNPs-mediated oxidative stress was associated with endocytosis. Endocytosis is a key route of cellular uptake of nanoparticles (Garcia-Alonso *et al.*,

2011). Cellular uptake of AgNPs via endocytosis contributed to ROS generation and stimulated genotoxic effects in human lung cells (Kim *et al.*, 2011a). The correlation between AgNP uptake and ROS generation also supports an oxidative mechanism for AgNPs' genotoxicity.

The ROS generation could result from mitochondrial dysfunction caused by AgNPs. AgNPs reduced ATP content and dehydrogenase activity, two indicators for mitochondrial damage (AshaRani *et al.*, 2009). AgNPs decreased the activities of the mitochondrial respiratory chain complexes I, II, III, and IV (Costa *et al.*, 2010) and altered mitochondrial membrane permeability, which disrupted mitochondrial functions, leading to ROS generation and oxidative stress (Teodoro *et al.*, 2011).

It has also been suggested that AgNPs may directly generate superoxide radicals by activating membrane-bound NAD(P)H oxidase during uptake into cells and then generating the hydroxyl radicals involved in the conversion of superoxide radicals to hydrogen peroxide (Kim *et al.*, 2011a), as previously described (Boelsterli, 2007).

3.3 Release of Silver Ions

Release of silver ions from AgNPs was confirmed by several different research groups (Kittler *et al.*, 2010; Lee *et al.*, 2012; Yang *et al.*, 2011). It was reported that AgNPs-induced genotoxicity was reduced by treatment with cysteine, a strong Ag^+ ligand, suggesting that silver ions released from AgNPs might contribute to the genotoxicity of AgNPs (Kawata, Osawa, and Okabe, 2009).

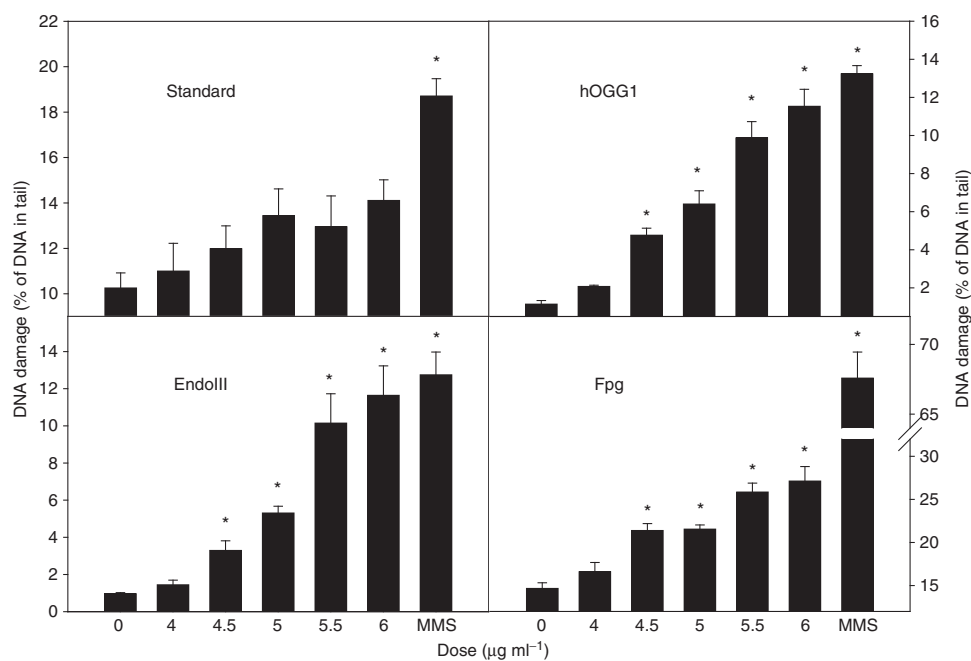


Figure 2. Oxidative DNA adducts induced by 5 nm AgNPs in mouse lymphoma cells using an enzyme-modified Comet assay (Mei *et al.*, 2012). (Permission has been obtained to use this figure from Copyright Clearance Center. License Number: 3143110782280). The Comet assay was performed with the standard procedure (top left panel), and addition of lesion-specific endonucleases: human 8-hydroxyguanine DNA-glycosylase (hOGG1, top right panel), endonuclease III (EndoIII, bottom left panel) and formamidopyrimidine DNA-glycosylase (FPG, bottom right panel) following treatment of L5178Y cells with different doses of 5 nm Ag-NPs. Methyl methanesulfonate (MMS) was used as the positive control. [Mei *et al.* (2012). Reproduced by permission of John Wiley & Sons, Inc.]

The released silver ions could interact with DNA directly and/or induce ROS (Arakawa, Neault, and Tajmir-Riahi, 2001). Silver ions released from AgNPs could interact with thiol groups in proteins and enzymes on the cell surface, inducing oxidative stress (Navarro *et al.*, 2008). Silver ions might also perturb the mitochondria through interactions with thiol groups in its inner membrane and cause mitochondria permeability transition pore (MPTP) (Almofti *et al.*, 2003). In addition, the ions released from AgNPs could deplete antioxidant defense mechanisms, thereby increasing oxidative stress (Chappell and Greville, 1954; Kone, Kaleta, and Gullans, 1988; Rangachari and Matthews, 1985).

4 FACTORS THAT AFFECT THE GENOTOXICITY OF SILVER NANOPARTICLES

Nanomaterials have unique physicochemical properties, and their characteristics contribute to their toxicity (Hoshino *et al.*, 2004; Magrez *et al.*, 2006; Oberdörster, 2004). Therefore, it is necessary to characterize nanomaterials before performing toxicological and genotoxicity studies (Landsiedel *et al.*, 2009; Oberdörster *et al.*, 2005; Oesch and Landsiedel, 2012; Powers *et al.*, 2006). Size effects, surface chemistry, and surface charge are the main properties that affect the genotoxicity of AgNPs.

4.1 Size Effects

Many studies have suggested that size is a critical factor for nanoparticle-induced toxicity and biological responses (Carlson *et al.*, 2008; Jia *et al.*, 2005; Jiang *et al.*, 2008; Nel *et al.*, 2006; Risom *et al.*, 2005). AgNPs with smaller size may have a larger fraction of their atoms on the surface, so surface reactivity increases as particle size decreases (Quadros and Marr 2010). The increasing surface reactivity may lead to higher toxicity. Li *et al.* (2012) found that 5 nm PVP coated AgNPs caused significant micronucleus induction in TK6 cells, whereas AgNPs with size greater than 5 nm in size only induced cytotoxicity but not genotoxicity.

4.2 Surface Chemistry

AgNPs have a tendency to aggregate and, therefore, are typically synthesized with surface coatings,

which maintain them in suspension (Kittler *et al.*, 2009; Liu *et al.*, 2010). The coating materials may change the surface chemistry of nanoparticles, causing them to be hydrophilic, hydrophobic, catalytically active, or passive. Thus, the surface chemistry of AgNPs can affect the interaction between nanoparticles and cells (Nel *et al.*, 2006). In some cases, the coating materials themselves are toxic or may function indirectly and enhance or inhibit cellular uptake, increasing or decreasing AgNPs toxicity. When AgNPs are coated with polysaccharide, a hydrophobic material, interactions between AgNPs and cells increase (Nel *et al.*, 2006) and produce more genotoxicity than uncoated AgNPs (Ahamed *et al.*, 2008). PVP, as a hydrophilic material, keeps the AgNPs less agglomerated and stable at their original size. Hence, PVP coated 5 nm AgNPs produce greater micronucleus induction than 5 nm uncoated AgNPs (Li *et al.*, 2012). However, some coatings may instead reduce the potential toxicity of nanomaterials (Lu *et al.*, 2010; Singh *et al.*, 2009).

4.3 Surface Charge

Surface charge also plays an important role in the interaction of AgNPs with cells such as cellular uptake. Generally, the plasma membrane is negatively charged because of the phospholipids on the outer surface. AgNPs with cationic surface charges appear to interact more actively with the membrane and generate greater cytotoxic responses as compared to those with anionic charges (El Badawy *et al.*, 2010). The negatively charged citrate-coated AgNPs were the least toxic, whereas the positively charged BPEI-AgNPs were the most toxic in bacillus bacterial cells. In addition, DNA is negatively charged, thus cationic nanoparticles are more likely to interact with this genetic material (Singh *et al.*, 2009).

5 PERSPECTIVES OF GENOTOXICITY STUDY ON SILVER NANOPARTICLES

Although a number of studies have demonstrated that AgNPs are genotoxic, the genotoxicity of AgNPs needs confirming. Many data gaps remain regarding the ability of AgNPs to induce DNA damage. Key issues for future studies are discussed as follows.

5.1 More Detailed and Complete Characterization of AgNPs

The unique properties of AgNPs make them different from bulk counterparts. It is important to include the characterization information during the genotoxicity assessment of AgNPs and establish the associations between genotoxic responses of AgNPs and their specific physicochemical properties. However, according to the available publications on genotoxicity of AgNPs, characterization on coating information and surface charge, as well as other characteristics of AgNPs, are lacking. Lack of complete characterizations makes it different to establish the possible association between those features and genotoxicity responses. In addition, pharmacokinetic analysis of AgNPs in animal studies is rare. Most of current available publications, especially those displaying negative results, fail to determine the concentration of AgNPs in target tissue such as bone marrow that is the target tissue for several *in vivo* genotoxicity assays. Without this information, it is difficult to know whether the negative results come from the negative response of AgNPs or because of lack of AgNPs in target tissues.

5.2 Application of Standard *In Vitro* and *In Vivo* Genotoxicity Assays

Although numerous publications reported the genotoxicity of AgNPs, only a few studies employed the standardized *in vitro* and *in vivo* assays. Thus, the results from different assays are not comparable. Standardized assays can provide advantages such as the same cell lines, similar exposure times, and same end points for direct comparison of results from different experiments and laboratories (Pfuhler *et al.*, 2013).

5.3 Understanding of Mechanisms Underlying Genotoxicity of AgNPs

The ultimate goal of genotoxicity study on AgNPs is to assess risks of AgNPs for human exposure. Currently available research results suggest that AgNPs induce genotoxicity via generation of ROS. Gene expression studies showed that expression of some genes related to oxidative stress was dysregulated by AgNPs treatment (Mei *et al.*, 2012),

and expression levels of other genes corresponding to metallothionein, heat shock protein, and histone families also were altered by AgNPs (Foldbjerg *et al.*, 2012). However, it is possible that multiple mechanisms of AgNPs existed and need to be investigated. Understanding all potential mechanisms of AgNP genotoxicity is necessary for regulatory agency to ensure impacts of the nanoparticles on human health.

RELATED ARTICLES

Chapter 1
Chapter 2

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Immunotoxicology of Nanomaterials

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1 INTRODUCTION

1.1 Nanomaterials

Nanomaterials, including semiconductor nanoparticles, nanotubes, nanoliposomes, and nanoemulsions, are of great potential use for many biological applications including molecular imaging (including targeted cancer imaging), live-cell labeling, RNAi delivery, and photodynamic therapy (Zhang *et al.*, 2007; Manzoor *et al.*, 2009; Sukhanova *et al.*, 2004; Chen *et al.*, 2005; Derfus *et al.*, 2007; Lee *et al.*, 2010; Zhang, Yee, Wang, 2008). Nanomaterials may also be bioconjugated and used for target-specific biological applications such as drug delivery (Akerman *et al.*, 2002). The use of nanomaterials for biological applications, including human therapeutic applications, is, however, dependent on their biocompatibility with living cells, including the cells of the immune system. Several factors have been found to modulate nanomaterial cytotoxicity, including size, charge, concentration, core composition, and capping agent molecular structure (Michalet *et al.*, 2005; Zhang, Yee, Wang, 2008). The basics of nanomaterial structure, composition, characterization, and application are reviewed elsewhere; here we focus on the biocompatibility of nanomaterials with the cells of the immune system.

1.2 The Immune System

Although we are constantly surrounded by potentially pathogenic microorganisms, we rarely become

ill because the cells and molecules of our immune systems have evolved complex systems of protection. The responses that our bodies mount against infection are termed *immune responses*, of which there are two main types, innate and adaptive immune responses. Innate immune responses are our first line of defense against infection, and are rapidly mounted, nonspecific responses to “danger.” Adaptive immune responses, in contrast, are highly specific responses that require days to weeks to develop, but that can lead to long-lasting protection against reinfection with the same pathogen. Most cells involved in innate immunity are derived from the common myeloid progenitor cells found in bone marrow; these cells include the macrophages, neutrophils, dendritic cells, monocytes, eosinophils, basophils, and mast cells. Unlike other inflammatory cells, tissue resident macrophages are cells that play several important roles in immune responses, including acting as our first responders to infection, effectively discriminating between “self” and “nonself” and producing and secreting a variety of signaling proteins, including cytokines and chemokines, to induce inflammation. The cells of the adaptive immune system are derived from common lymphoid progenitor cells in the bone marrow and include the T lymphocytes, the B lymphocytes, and the natural killer (NK) T cells.

Inflammation is a response to infection traditionally defined by four Latin words: calor, dolor, rubor, and tumor (heat, pain, redness, and swelling) (Murphy, Travers, Walport, 2012). Several important events occur during inflammatory responses

that allow the cells of the immune system to fight infection. The signaling proteins secreted by macrophages trigger a phenomenon known as *endothelial activation*, a process that results in blood vessel dilation, increased vascular permeability and increased adherence of circulating white blood cells (including neutrophils, monocyte, and lymphocytes) to blood vessel walls. The macrophage-derived signaling molecules also recruit the adhered white blood cells out of circulation and into the tissue at the site of infection. The first class of white blood cells recruited to the submucosal tissue is the neutrophils, with the influx of neutrophils peaking by 6 h after the initial pathogenic insult. The movement of neutrophils (and other white blood cells) out of circulation and into the tissue in response to macrophage-derived signaling molecules is called *extravasation* and consists of four basic steps: rolling adhesion of neutrophils to the endothelium, tight binding of neutrophils to endothelial cells, diapedesis (migration of neutrophils between adjacent endothelial cells), and migration of neutrophils along a concentration gradient of macrophage-produced signaling molecules to the site of infection. Once in the tissue, neutrophils aid macrophages in the identification and clearance of the invading microorganism, and as the inflammatory process progresses, additional macrophages may also be recruited to the site of infection (Pober, 2002; Murphy, Travers, Walport, 2012). If the cells of the innate immune system are unable to resolve an infection, the lymphocytes of the adaptive immune system may be recruited out of circulation by macrophage-produced signaling molecules. The antigen-specific activation of T lymphocytes or B lymphocytes triggers both a targeted response to pathogenic insult and the generation of long-lived memory cells that are uniquely primed to protect against reinfection.

While the activity of inflammatory cells is vital for host response to infection, inappropriately high or prolonged activity results also in host tissue destruction. Furthermore, it has become increasingly clear that inflammatory responses not only occur in response to infection but also may be inappropriately mounted against host tissue and/or inherently noninfectious stimuli. Inappropriate and/or dysregulated immune responses are a major cause of morbidity and mortality in many common diseases including stroke,

heart attack, sickle cell anemia, sepsis, colitis, autoimmunity, and allergy. Because of the prevalence of inflammation as a modulator of human health, the safe and efficacious *in vivo* use of any nanomaterial is inherently linked to a favorable interaction with immune system cells; immune system recognition and clearance must be avoided, as must any untoward effects on immune system function.

1.3 The Biocompatibility of Nanomaterials with Immune System Cells

The clinical, biological, or industrial use of nanomaterials is dependent on their biocompatibility with human cells, including the cells of the immune system. Studies have shown that nanomaterials with varying core and outer shell compositions elicit variable effects on immune cell viability and function—with both direct and indirect cytotoxic effects elicited by exposure to select nanomaterials. Nanomaterial exposure has been demonstrated to trigger inflammatory responses, induce oxidative stress and leukocyte apoptosis, alter macrophage metabolism, and modulate white blood cell proliferation (Hanini *et al.*, 2011; Srinivas *et al.*, 2011; Chen *et al.*, 2010; Liu *et al.*, 2011). Furthermore, nanomaterial exposure can trigger systemic T lymphocyte activation, induce pro-inflammatory cytokine production by T lymphocytes, and result in T lymphocyte death (Park, Choi, Park, 2011a; Ying and Hwang, 2010; Zhu *et al.*, 2012). In addition to their observed immunomodulatory effects, nanoparticles have also been shown to accumulate in the spleen, with long-term exposure resulting in chronic spleen injury (Almeida *et al.*, 2011; Sang *et al.*, 2012). Two major pathways of nanomaterial cytotoxicity have been suggested; the dissolution and release of toxic metals and toxic effects associated with nanoparticle properties such as size and surface chemistry (Pace, Leshner, Ranville, 2010). A review of the effects of nanomaterial exposure on the immune system follows; contained within are discussions of the immunomodulatory effects of several of the most widely used nanomaterials including carbon nanotubes, titanium nanoparticles, zinc nanoparticles, silica nanomaterials, and silver nanoparticles.

2 MAIN TEXT

2.1 Carbon Nanotubes

Single-walled carbon nanotubes (SWCNTs) and multi-walled carbon nanotubes (MWCNTs), because of their unique structural and functional properties, possess broad potential for use in industrial and medical applications. Carbon nanotubes are readily internalized by various mammalian cells, making them attractive tools for use in biological imaging as well as drug and gene delivery. The facility of carbon nanotubes to enter mammalian cells, along with the increasing utilization of these nanomaterials in technological, medical, and consumer products, underscores the importance of a thorough investigation of the toxicological impact of carbon nanotube exposure. Several groups have investigated the impact of SWCNT and MWCNT exposure on immune system cells, with accumulating evidence suggesting that carbon nanotube exposure modulates immune system function.

The inhalation of both SWCNTs and MWCNTs has been found to elicit detrimental pulmonary effects mediated by the induction of inflammation, chronic granulomatous lung disease, fibrosis, or, conversely, immunosuppression (Huizar *et al.*, 2011; Inoue *et al.*, 2009; Wang *et al.*, 2011b). In both mouse and rat models, SWCNT inhalation has been shown to trigger a robust recruitment of neutrophils, macrophages, and lymphocytes to the site of nanotube exposure, with neutrophil accumulation peaking 24 h post inhalation, lymphocyte infiltration peaking 3 days post inhalation, and macrophage influx peaking 7 days post exposure. The infiltration and accumulation of leukocytes in pulmonary tissue is accompanied by enhanced levels of the pro-inflammatory cytokines TNF- α and IL-1 β . In addition, lactose dehydrogenase and gamma-glutamyl transferase activities (markers of inflammation) are elevated in bronchoalveolar lavage fluid after SWCNT exposure, and an accumulation of 4-hydroxynonenal (an oxidative biomarker) and depletion of glutathione in lung tissue is observed. These pro-inflammatory effects of SWCNT inhalation lead to a rapid, progressive lung fibrosis and granuloma formation in both mouse and rat models. SWCNT-induced fibrosis exhibits two distinct morphologies: SWCNT-induced granulomas associated predominantly with hypertrophied

epithelial cells surrounding SWCNT aggregates, and diffuse interstitial fibrosis and alveolar wall thickening likely associated with dispersed SWCNT (Shvedova *et al.*, 2005).

Similar to the effects elicited by SWCNT inhalation, MWCNT exposure induces pulmonary and systemic alterations in immune system activity (Katwa *et al.*, 2012; Mitchell *et al.*, 2007; Park *et al.*, 2009a). The total number of immune cells in bronchoalveolar lavage fluid of mice after intratracheal instillation of 5, 20, or 50 mg kg⁻¹ doses of MWCNTs is significantly increased as early as 24 h after administration. Correspondingly, the pro-inflammatory cytokines IL-1, TNF- α , IL-6, IL-4, IL-5, IL-10, IL-12, and IFN- γ also increase in a dose-dependent manner both in BAL fluid and in blood. Interestingly, TH2 cytokines are elevated to a greater degree than TH1 cytokines, but levels of all pro-inflammatory cytokines peak at 24 h after MWCNT instillation and then gradually decrease. In addition, the numbers of B lymphocytes in both the spleen and the blood increases significantly 24 h after nanotube administration, perhaps resulting directly from elevated circulating levels of the B cell-activating TH2 cytokines. Accompanying the increases in leukocyte number and cytokine production, MWCNT administration triggers a dose-dependent granuloma formation in lung tissue (Park *et al.*, 2009a). Notably, it has been demonstrated that the toxicological and immunological effects of MWCNT inhalation are observed only in mice with functional mast cells. Mast cells, which play a role in both innate and adaptive immune responses, are known to respond to danger through the activation of a variety of cell surface receptors, including the IL-33-activated IL-1-like receptor, ST2. Mice deficient in mast cells, or possessing mast cells incapable of responding to IL-33, are protected from the immunomodulatory effects of MWCNT exposure, suggesting that mast cells and the IL-33/ST2 axis orchestrate adverse pulmonary and immunological responses to carbon nanotubes (Katwa *et al.*, 2012).

Although exposure to SWCNTs and MWCNTs can undoubtedly mediate pro-inflammatory responses, several studies have demonstrated that carbon nanotube exposure can also (or instead) result in immunosuppression. When mice are exposed via whole-body inhalation to 0.3–5 mg m⁻³ respirable aggregates of MWCNTs, systemic alterations in immune system activity are observed.

Histopathology of lungs from exposed animals, as well as BAL fluid, shows particle-laden alveolar macrophages, and exposure to 0.3 mg m^{-3} and higher particle concentrations causes systemic immunosuppression after 14 days, but not at earlier time points. MWCNT-induced immunosuppression is characterized by a reduced T-cell-dependent antibody response as well as reduced T-cell proliferative activity in the presence of the mitogen, Concanavalin A. Assessment of nonspecific NK cell activity indicates that animals exposed to 1 mg m^{-3} MWCNTs have impaired NK cell function as well as elevated mRNA levels of IL-10 and NADPH oxidoreductase 1 (Mitchell *et al.*, 2007). When mice are exposed to MWCNTs for 6 h per day for 14 consecutive days in whole-body inhalation chambers, marked immune system suppression is observed, and is correlated with activation of cyclooxygenase enzymes in the spleen in response to a signal from the lungs. Spleen cells from exposed animals partially recover their immune function when treated with ibuprofen, a drug that blocks the formation of cyclooxygenase enzymes, whereas mice with nonfunctional cyclooxygenase enzymes experience no immunosuppression when exposed to MWCNTs (Mitchell *et al.*, 2009). These data suggest that the immune system suppression elicited by MWCNT exposure is mediated, at least in part, by cyclooxygenase activity, although the precise mechanism of suppression is unknown.

Taken together, results from studies investigating the effects of *in vivo* exposure to SWCNTs and MWCNTs suggest that these nanomaterials induce a preliminary, acute, pro-inflammatory response that progresses over time to an immunosuppressive phenotype. This hypothesis is supported by evidence from *in vitro* studies aimed at elucidating the mechanisms of carbon nanotube-elicited immune system modulation. Because immune system activity is reliant on complex interactions between cells and molecules, targeted *in vivo* and *in vitro* studies rarely provide a complete picture of immunoregulatory pathways. To better characterize the broad effects of carbon nanotube exposure on immune system function, genome-wide microarray studies have been utilized to analyze the effects of MWCNT exposure on THP1 monocytic cells and Jurkat T cells—cell lines that are representative of the innate and adaptive immune responses respectively. After analysis of more than 32 000 transcripts, it has been found that MWCNTs activate

immune-related pathways largely in monocytes than in T cells; molecular pathways upregulated by MWCNT exposure include IL6, CD40, dendritic cell maturation, TNF- α /TNFR1-2, NF- κ B signaling, and TH1 chemokine pathways (CXCR3 and CCR5 ligand pathways). These pathways are commonly activated during acute inflammatory processes, indicating the MWCNTs can act as cell-specific immunostimulatory agents (Pescatori *et al.*, 2013). Interestingly, independent studies have demonstrated that the *in vitro* exposure of murine macrophages to SWCNTs triggers the production of the anti-inflammatory cytokine TGF- β , inhibits the production of the pro-inflammatory cytokines TNF- α and IL-1 β , and suppresses the pathogen-induced macrophage respiratory burst (Shvedova *et al.*, 2005). Furthermore, SWCNTs with low content of metal impurities, under noncytotoxic exposure conditions, suppress the chemotaxis of primary human monocytes and impair macrophage engulfment of apoptotic target cells (Witasp *et al.*, 2009b). These data once again underscore the varying immunomodulatory effects of nanotube exposure.

Although multiple studies have confirmed that carbon nanotubes are readily internalized by macrophages, B lymphocytes, and T lymphocytes, varying effects on cell viability have been observed. Functionalized carbon nanotubes prepared via a 1,3-dipolar cycloaddition reaction or an oxidation/amidation treatment, although internalized, do not affect monocyte, macrophage, T cell, B cell, or NK cell viability; smaller diameter nanotubes are more efficiently internalized by all cell types. Although the functionalized carbon nanotubes have been observed to be nontoxic under low dose conditions, monocyte, macrophage, and NK cell functionality are influenced, namely via the induction of pro-inflammatory cytokine production (Dumortier *et al.*, 2006; Delogu *et al.*, 2012). In contrast to these findings, pristine and oxidized MWCNTs have been demonstrated to induce massive loss of T lymphocyte cell viability through programmed cell death at doses of $400 \mu\text{g ml}^{-1}$. Lower concentrations of pristine and oxidized MWCNTs are significantly less toxic, illustrating that the cytotoxicity of the nanomaterials is dose-dependent (Bottini *et al.*, 2006). Recent studies suggest that reactive oxygen species (ROS) induced in mammalian cells exposed to MWCNTs could mediate the cytotoxicity of the

nanomaterials. Indeed, high levels of ROS and decreased metabolic activity are observed in Jurkat T cells after acute exposure to high concentrations (up to $30 \mu\text{g mL}^{-1}$) of MWCNTs (Thurnherr *et al.*, 2011). Similarly, treatment of primary human macrophages with $100 \mu\text{g mL}^{-1}$ MWCNTs induces a time-dependent decrease in cell viability and an increase in intracellular superoxide levels that is correlated with the membrane translocation of the NADPH oxidase subunits p47phox and p67phox, a marker of NADPH oxidase activation. The pretreatment of macrophages with apocynin, a selective inhibitor of NADPH oxidase, prevents the MWCNT-induced membrane translocation of p47phox, the elevation of superoxide levels, and the decrease in cell viability (Ye *et al.*, 2011). These findings demonstrate that MWCNTs activate NADPH oxidase in human macrophages, which may contribute to ROS generation and cell death in MWCNTs treated-macrophages; similar mechanisms of carbon nanotube-induced cytotoxicity may occur in other inflammatory cells as well.

2.2 Titanium Nanoparticles

Owing to their high stability, anti-corrosiveness, surface activity, and photocatalytic properties, titanium dioxide nanoparticles (TNPs) are among the most widely manufactured nanomaterials. The broad utilization of TNPs in various applications, including cosmetics, pharmaceuticals, and industrial purposes, is correlated with the increasing risks of environmental and/or occupational exposure; multiple studies have been aimed at investigating the effects of such exposure on immune system activity and viability. The *in vivo* administration of TNPs has been demonstrated to have multiple immunomodulatory effects, characterized by alterations in immune cell number, viability, and function. The intratracheal instillation of $0.5\text{--}50 \text{ mg kg}^{-1}$ of TNPs results in multiple, and often inconsistent, alterations in immune system activity. For example, levels of the pro-inflammatory cytokines IL-1, TNF- α , and IL-6 are significantly elevated in a dose-dependent manner as early as 24 h after TNP administration, and remain elevated for up to 14 days; levels of the TH1 cytokines IL-12 and IFN- γ and the TH2 cytokines IL-4, IL-5, and IL-10 are also elevated dose-dependently at day 1 and remain elevated for up to 14 days after TNP exposure. Perhaps resulting

from the elevation in TH2 cytokine levels, increased numbers of B lymphocytes are observed in both spleen and in blood, and increased IgE production in BAL fluid and serum is observed. Furthermore, an increase in the inflammatory proteins MIP and MCP, as well as granuloma formation, are observed in lung tissue after the intratracheal administration of TNPs (Park *et al.*, 2009b). These data suggest that exposure to TNPs has the potential to cause chronic inflammatory disease. In contrast to these findings, however, it has been shown that the inhalation of TNPs can elicit immunosuppressive effects. The intratracheal administration of TNPs can trigger membrane and ultrastructure damage of pulmonary alveolar macrophages, as well as alterations in macrophage phagocytic ability; the phagocytic activity of macrophages is increased after exposure to low doses of TNPs, but decreased after exposure to high doses. Exposure to TNPs also has been found to decrease the chemotactic ability of alveolar macrophages as well as the expression of Fc receptors and MHC-class II on the macrophage cell surface (Liu *et al.*, 2010). These data indicate that TNP exposure may reduce immune system activity via damage to immune cell structure and activity. The seemingly incongruous effects of *in vivo* TNP exposure on immune system function are illustrated by the observation that the intragastric administration of TNPs causes both an increase in the mRNA and protein expression of the pro-inflammatory TLR2, TLR4, NF- κ B, and TNF- α , as well as the significant decrease in the mRNA and protein expression of the potent pro-inflammatory mediator, IL-2 (Cui *et al.*, 2011).

To more fully characterize the effects of TNP exposure, whole-genome microarray analysis has been utilized to examine the gene expression profiles of mice exposed to 10 mg kg^{-1} TNPs for 90 days. TNP exposure triggers striking changes in the expression of 785 genes related to the immune response—including genes involved in apoptosis, oxidative stress, metabolic processes, response to stress, ion transport, signal transduction, cell proliferation, and cell differentiation. In particular, a significant reduction in complement factor D expression is observed, and it results in autoimmune and inflammatory disease states in mice (Cui *et al.*, 2012). These data underscore the ability of TNPs to modulate multiple aspects of immune function; the potentially opposing effects elicited by various TNP exposure conditions may be due to a dose

and/or time dependence, but this has not been determined unequivocally. Although there exists some ambiguity surrounding the immunomodulatory effects of TNP exposure, it is better established that TNPs can accumulate in lymphoid organs, with the potential to cause tissue damage and/or dysfunction. Intravenously administered TNPs accumulate in the spleen, with levels peaking 24 h after administration and decreasing only slightly by days 14 and 28 (Fabian *et al.*, 2008). Similarly, when administered via intragastric exposure, TNPs cause congestion and lymph nodule proliferation of spleen tissue, with accompanying increases in ROS levels in spleen tissue. The elevated ROS levels in affected spleens lead to lipid peroxidation and an upregulation of heme oxygenase-1 expression via the p38-Nrf-2 signaling pathway, suggesting that TNP accumulation in lymphoid organs may exert cytotoxic effects through the induction of oxidative stress (Wang *et al.*, 2011a).

Owing to the sometimes (seemingly) opposing effects of *in vivo* TNP exposure on immune cells, *in vitro* studies have been utilized to more clearly investigate the mechanisms of TNP immunomodulatory activity. Treatment of peripheral blood mononuclear cells (PBMCs) with TNPs leads to a dose-dependent elevation of the pro-inflammatory cytokines IL-1 α , IL-1 β , IL-6, IL-8, IL-10, IFN- γ , and TNF- α , as well as an upregulated expression of the maturation marker CD83 and the co-stimulatory molecule CD86 on dendritic cells. Notably, 48 h of TNP exposure also results in a 10–20-fold increase in ROS levels in PBMCs (Schanen *et al.*, 2009). The direct exposure of microglia (a tissue-specific macrophage-like cell) to TNPs results in a significant increase in iNOS expression, along with a correlated increase in NO release from the microglia. The expression levels of MCP-1 and MIP-1 α are also upregulated, as is the activation of NF- κ B. In addition, the secretion of TNF- α , IL-1 β , and IL-6 is increased (Xue, Wu, Sun, 2012). Further, demonstrating the immunomodulatory activities of direct TNP exposure, it is known that TNPs induce the expression of the inflammasome NLRP3 by dendritic cells and macrophages—an event that leads directly to the activation of caspase-1 and the subsequent release of the mature form of IL-1 β (Sun *et al.*, 2012; Winter *et al.*, 2011). Taken together, these data are consistent with other findings suggesting that TNPs induce immune cell activation and the subsequent release of pro-inflammatory

factors. However, TNP exposure also possesses the potential to cause immune cell death. The exposure of dendritic cells to TNPs induces a dose-dependent decrease in cell viability, with higher concentrations of TNPs eliciting an approximate 20% decline in viability (Schanen *et al.*, 2009). Human dermal fibroblasts capable of exhibiting inflammatory responses also exhibit a time- and dose-dependent decrease in cell viability on exposure to TNPs, with a corresponding increase in ROS levels and expression of several pro-inflammatory genes including the TLRs, genes of the MAP kinase cascade and IL-1 β . Notably, several mediators of the NF- κ B pathway are activated: ERK1/2 expression is upregulated and p38 is phosphorylated. Interestingly, NF- κ B knock-down fibroblasts are resistant to TNP-induced cell death, suggesting that the activation of the NF- κ B pathway plays a role in TNP-induced immune cell death (Romoser *et al.*, 2012).

2.3 Zinc Nanoparticles

The characteristic physical and optical properties of zinc nanoparticles, including their size, intense fluorescence, broad excitation, narrow emission, and resistance to photobleaching, make these nanomaterials potentially useful for a variety of biological applications including molecular imaging, live-cell labeling, photodynamic therapy, and targeted drug delivery. However, several reports suggest that these metal nanoparticles possess cytotoxic potential. Studies show that ZnO nanoparticles are efficiently internalized by PBMCs, and after 24 h of exposure, a dose-dependent increase in both active caspases and DNA fragmentation is observed with as many as 84% of cells undergoing apoptosis after 24 h. Interestingly, at 24 h after exposure, the ZnO nanoparticles are not detected within the vesicles of some apoptotic cells, suggesting that particle dissolution has occurred, and may potentially contribute to the cytotoxic effects of the nanomaterials via the release of zinc ions (Andersson-Willman *et al.*, 2012). Consistent with this observation, it has been demonstrated that ZnO nanoparticle-induced Jurkat T cell death is largely an ionic effect involving the release of high amounts of zinc ions. ZnO nanoparticles are rapidly internalized by T cells and trigger a caspase-independent alternative apoptosis pathway that is independent from the formation of ROS. The cytotoxicity of ZnO nanoparticles is reduced

by the addition of coating agents that minimize ZnO nanoparticle dissolution (Buerki-Thurnherr *et al.*, 2012). It is clear from the results of several reports that the dissolution of zinc nanoparticles plays at least a partial role in their toxicity, but it has also been established that the cytotoxicity of the nanomaterials is dependent on a direct cell contact. Coupled with the fact that zinc nanoparticles are readily internalized by immune cells, it is likely that on zinc nanoparticle internalization, free metal ions are released within lysosomal compartments, delivering an intracellular dose of zinc that is higher than can be achieved by extracellular exposure to dissolution of metal nanoparticles (Cho *et al.*, 2011; Moos *et al.*, 2010; Studer *et al.*, 2010). Although zinc is an essential trace element in humans, an excess of zinc ions is cytotoxic and can trigger apoptosis in some cell types (Watjen *et al.*, 2002).

The cytotoxicity of zinc nanoparticles is dependent not only on dissolution rate but also on particle size, charge, solubility, and capping agent structure. ZnO nanoparticles with an average diameter of greater than 70 nm exhibit enhanced cytotoxicity, and a positive charge enhances this cytotoxicity (Prach, Stone, Proudfoot, 2013). Furthermore, the biocompatibility of ZnS nanoparticles with murine splenocytes is dependent on both the chemical structure of the outer capping agent as well as the duration of time in suspension before cell exposure. Exposure of isolated splenocytes to 1–10 mg ml⁻¹ of freshly resuspended ZnS nanoparticles capped with glutamic acid or cysteine results in a significant reduction in cell viability, whereas L-histidine, L-isoleucine, tryptone, polyphosphate, peptone, and PVP-capped ZnS nanoparticles exhibit no cytotoxic effects when cultured with murine splenocytes at concentrations ranging from 1 to 10 mg ml⁻¹ (Weilnau *et al.*, 2013). Notably, although both freshly resuspended and 2-day-old tryptone, polyphosphate, and peptone-capped ZnS nanoparticles have no significant effect on splenocyte viability, 9-day-old nanoparticles significantly reduce cell viability after 24 h of exposure. More strikingly, after 14 days in suspension, tryptone, polyphosphate, and peptone-capped nanoparticles all reduce cell viability by approximately 50% when added to splenocyte cultures. Similarly, after 14 days in suspension, L-histidine and L-isoleucine-capped nanoparticles also significantly reduce cell viability. The exposure of murine splenocytes to

nanoparticle-free supernatants collected from tryptone, polyphosphate, peptone, L-histidine, or L-isoleucine-capped ZnS nanoparticle suspensions at time points ranging from 0 to 14 days after nanoparticle resuspension results in cell death that is not significantly different than vehicle treated controls. These data indicate that the increased cytotoxicity of the aged nanoparticle suspensions is not a result of nanoparticle dissolution or any accompanying release of toxic levels of zinc into the suspension (Weilnau *et al.*, 2013).

In addition to possessing cytotoxic potential, zinc nanoparticles, at sublethal doses, have immunomodulatory activity. ZnO nanoparticles influence the activation of the human monocytic cell line THP1 and induce a modest increase in the expression of the adhesion molecule CD11b (Prach, Stone, Proudfoot, 2013). In addition, ZnO nanoparticle exposure triggers the enhanced production of the pro-inflammatory cytokine IL-1 β and the neutrophil chemoattractant CXCL-9 by both RAW 264.7 murine macrophages and murine bone-marrow-derived dendritic cells (Palomaki *et al.*, 2010).

2.4 Silica Nanomaterials

Silica nanoparticles are attractive nanomaterials for use in biomedical applications including use as drug delivery systems. However, silica-based nanomaterials possess numerous immunomodulatory activities. The pore structure of silica nanomaterials is thought to influence cytotoxicity because of its influence on cellular uptake and immune response. Mesoporous silica nanomaterials differ significantly from general colloidal silica in regard to pore architecture, including surface area and pore volume, making them valuable models to use in the investigation of the effects of pore structural conditions of silica nanoparticles on inflammation and immune cell viability. When analyzed via 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) assay or fluorescent activated cell sorting (FACS) analysis, mesoporous silica nanoparticles induce significantly less murine macrophage apoptotic cell death than do colloidal silica nanoparticles. In addition, mesoporous silica nanoparticles induce a less robust production of TNF- α , IL-1 β , and IL-6 by macrophages than do colloidal silica nanoparticles.

Correspondingly, exposure of murine macrophages to mesoporous silica nanoparticles induces a lesser activation of the MAP kinase pathway, NF- κ B, and caspase 3 than does exposure to colloidal silica nanoparticles, a likely mechanism for the reduced pro-inflammatory and cytotoxic effects observed. It has also been observed that colloidal silica, but not mesoporous silica, nanoparticles act as immunogenic sensitizers that induce contact hypersensitivity, further establishing that the pore architecture of silica nanoparticles significantly influences their biocompatibility, with mesoporous silica nanoparticles exhibiting much greater biocompatibility than colloidal silica nanoparticles (Lee, Yun, Kim, 2011). Interestingly, although mesoporous silica nanoparticle exposure does not significantly affect primary macrophage viability or function, monocyte-derived macrophages readily and efficiently internalize the nanoparticles via endocytosis (Witasap *et al.*, 2009a). Likewise, both nonporous and mesoporous silica nanoparticles are internalized by primary mast cells, localizing primarily in the secretory granules, with uptake efficiency greater for the mesoporous than the nonporous nanoparticles. Assessment of the influence of nanoparticle-laden granules reveals functional changes in chemical messenger secretion from mast cell granules; both nonporous and mesoporous silica nanoparticles cause a decrease in the number of molecules released per granule, with nonporous particles having a larger impact on cell function (Maurer-Jones, Lin, Haynes, 2010).

In addition to pore architecture, size and surface functionality also influence silica nanoparticle biocompatibility. The cellular internalization, cytotoxicity, and immune response of murine alveolar macrophages to silica-titania hollow nanoparticles with diameters of 25, 50, 75, 100, or 125 nm and cationic, anionic, or neutral functional group surfaces have been investigated; 50 nm nanoparticles with cationic surface functionality are internalized with the greatest efficiency, elicit the most robust production of IL-1, IL-6, and TNF- α , trigger the highest levels of ROS production, and induce the greatest rates of cellular apoptosis. The apoptosis elicited by silica-titania nanoparticles is attributed to the perturbation of proton pump activity, leading to lysosomal rupture or damage to the mitochondrial outer membrane resulting in the release of pro-apoptotic factors (Oh *et al.*, 2010). Furthermore, when

administered intravenously, silica nanoparticles accumulate in the spleen, with histological evaluation revealing a corresponding infiltration and hyperplasia of macrophages in the spleen resulting in overall physical spleen enlargement (Yu *et al.*, 2012). These data underscore the importance of considering architecture, size, and surface functionality when evaluating silica nanoparticle biocompatibility.

2.5 Silver Nanoparticles

Silver nanoparticles are of increasing interest for use in medical settings because of their well-established antimicrobial properties. Despite the relatively widespread use of silver nanomaterials, a thorough investigation of their biocompatibility with immune system cells has yet to be undertaken. Multiple studies have, however, demonstrated that nanosilver products possess potentially immunotoxic and/or immunomodulatory activities. Exposure to silver nanoparticles causes significant immunotoxicity in human PBMCs as well as in isolated monocytic cells. PBMCs and THP-1 monocytic cells efficiently internalize silver nanoparticles and experience a time- and dose-dependent decrease in cell viability on exposure. In addition, nanosilver induces a reduction of NO production and an upregulation of the expression of Myd88, MEKK1, and early regulation of oxidative stress genes, in both PBMCs and THP-1 cells (Hayashi *et al.*, 2012). Similarly, exposure for 24, 48, or 72 h to 1–25 ppm silver nanoparticles causes a significant decrease in the cell viability of murine peritoneal macrophages, and exposure to 0.4–25 ppm of silver nanoparticles results in a significant decline in NO production by murine macrophages (Shavandi, Ghazanfari, Moghaddam, 2011). Nanosilver is also known to induce apoptosis in fibroblasts capable of exhibiting immune responses. Exposure of fibroblasts to silver nanoparticles triggers the release of cytochrome c into the cytosol and the translocation of Bax to mitochondria, indicating that nanosilver-mediated apoptosis is mitochondria-dependent. Nanosilver-induced apoptosis in fibroblasts is correlated with an increase in ROS production and c-Jun N-terminal kinase (JNK) activation; the inhibition of either ROS production or attenuation of nanosilver-induced apoptosis by JNK (Hsin *et al.*, 2008). It has also been shown that silver nanoparticles cause leakage of cathepsins

from lysosomes and efflux of intracellular potassium ions in human blood peripheral mononuclear cells; these events induce superoxide production within mitochondrial membranes leading to inflammatory NLRP3 formation and a resulting increase in IL-1 β production. Although both 5 nm and 28 nm silver nanoparticles cause an increased mitochondrial production of superoxide in PBMCs and monocytic cells, 5 nm nanoparticles induce significantly greater production, which is correlated with decreased cell viability (Yang *et al.*, 2012). A size-dependent effect of silver nanoparticle exposure is also observed in RAW 264.7 murine macrophages, with silver nanoparticles with a 20 nm diameter inducing significantly more apoptotic cell death, ROS production, decrease in metabolic activity, and production of G-CSF, TNF- α , IL-1 α , IL-1 β , IL-6, MIP-1 α , MIP-1 β , and MIP-2 than nanoparticles with 80 nm or 113 nm diameters (Park *et al.*, 2011b). A more thorough elucidation of the size-dependent effects of silver nanoparticle exposure on human macrophages has been accomplished using cDNA microarray analysis. A total of more than 28 000 cDNA profiles have been screened after macrophage exposure to sublethal concentrations of 5 nm or 100 nm silver nanoparticles to determine if the changes in induced gene expression differ; it is evident that exposure to 5 nm silver nanoparticles results in a more robust expression of pro-inflammatory cytokines (including IL-8) and stress genes (including hemeoxygenase-1 and heat shock protein-70), as compared to exposure to 100 nm silver nanoparticles (Lim *et al.*, 2012).

3 CONCLUSION/SUMMARY

Owing to their characteristic physical, chemical, and optical properties, nanomaterials are being studied for use in a wide range of biological and therapeutic applications. However, multiple nanomaterials, including the widely used carbon nanotubes, titanium nanoparticles, zinc nanoparticles, silica nanomaterials, and silver nanoparticles, have demonstrated potential to exert immunomodulatory and immunotoxic effects. The biocompatibility of nanomaterials with immune system cells is dependent on their surface chemistry, core composition, pore architecture, size, concentration, and chemical nature of the outer capping agent; nanomaterial

cytotoxicity is also related to efficiency of cell internalization, induced ROS production and influence of nanomaterial exposure on gene expression profiles. As nanomaterials are introduced into pharmaceutical, commercial, and/or industrial products, it is vital that their effects on human health and immune system function are carefully evaluated.

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Neurotoxicity of Nanoparticles

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1 INTRODUCTION

Particulate materials have always been present in the atmosphere. In the past, these were largely organic materials such as pollen and various plant fragments. Carbonaceous materials originating from forest fires were another intermittent component. Inorganic wind-borne dusts such as fine sand and dried ocean salt could also be found. The advent of modern industrial society has greatly expanded the number of substances found in some air samples. Airborne particulates can originate from all of the many combustion processes that prevail in contemporary society. It is well known that such particulates can be injurious to the lung and that the nature of the damage incurred is related to both the physical and the chemical nature of the inhaled materials. Thus, the hazard of lung damage has increased in modern times especially in the growing urban population.

The functioning of other organ systems, notably the cardiovascular system, has been shown to be affected by the inhalation of particulates. In view of the proximity of the vasculature and the alveoli of the lung, and the key role of pulmonary gas exchange, this is perhaps not surprising. However, the ability of inhaled solids to have a deleterious effect on the central nervous system (CNS) is perhaps more unexpected as the mature brain has a sophisticated defensive system designed to protect it from many agents that are present found in the circulation, that are potentially harmful to the brain.

There have been many recent reviews on the neurotoxicity of particulates and this review is focused on certain areas that have not hitherto been emphasized (Simkó and Mattsson, 2010). The puzzle of the means by which nanoparticles, <100 nm in size, may have access to the brain is addressed initially. The types of damage that have been reported as a consequence of such entry are then described. This leads to the broader question of whether some types of neurological disease may owe their occurrence or progression to such particulates. Finally, it is important to understand the possible mechanisms underlying the neurotoxicity of nanoparticles. The degree of reversibility and possible treatments following exposure to nanoparticles and expression of neurotoxic signs, are also relevant (Yang *et al.*, 2010).

2 SOURCES OF NANOPARTICLES

Nanoparticles found in urban air are largely derived from incomplete combustion of fossil fuels. These include products derived from coal, gasoline, and diesel oil. Such particles, while largely carbonaceous, also contain metals. These mixtures of organic compounds together with inorganic metal oxides can be toxic in ways that cannot be predicted by study of individual components in isolation. More specific types of ultrafine particle containing a less complex spectrum of ingredients may be encountered in industrial settings such as metal smelting and mining, in various medical,

agricultural and cosmetic preparations. While all materials have the potential for causing selective damage to the target tissue, especially the lung in the case of inhaled materials; access to the brain is less ubiquitous. More recently, engineered nanomaterials are becoming common in textiles used for clothing manufacture and in façade coatings used on wall coverings (Som *et al.*, 2011). The unintended release of nanoparticles from these materials can broaden the population range and intensity of those exposed nanoparticles.

3 MEANS OF ENTRY OF NANOPARTICLES INTO THE BRAIN

3.1 The Olfactory Pathway

The olfactory pathway as a means of delivery of large molecules and nanoparticles to the brain has been established recently. Bodian and Howe described the transport of the poliovirus by this means as long ago as 1941. De Lorenzo 1970 showed that colloidal gold particles could move from the olfactory epithelium to the olfactory bulb. Such entry into the brain may be by way of olfactory nerve transport of deposits from the nasal olfactory mucosa (Oberdörster *et al.*, 2004). The degree of entrapment of materials by the nasal mucosa is very dependent on particle size. After nose-only inhalation, ultra-fine particles with a diameter of <5 nm, are almost completely retained by the nasal mucosa (Keck *et al.*, 2000).

3.2 Other Nerve Pathways of the Respiratory Tract

Another possible mechanism is transport along the sensory trigeminal nerves into the CNS (Mistry, Stolnik, and Illum, 2009, Hunter and Dey, 1998). Nanoparticles appear to be taken up by various termini of the trigeminal and other sensory nerves located at several sites in the respiratory tract including the nasopharyngeal and tracheobronchial regions (Oberdörster, Elder, and Rinderknecht, 2009). Such entry by endocytotic particle uptake and the presynaptic nerve termini followed by retrograde axoplasmic transport along neuronal tracts, can circumvent the blood–brain barrier (BBB) and permit nanoparticles to enter the CNS.

3.3 Transport through the Blood–Brain Barrier

The BBB may be traversed or circumvented by several means.

3.3.1 Receptor Mediated Endocytosis

The transfer of specific molecules into the brain can take place by their binding to a surface on the inner lining of capillaries followed by transport across the capillary epithelium and endocytotic release into the brain tissue. This physiological process may be hijacked by nanoparticles. The ability of nanoparticles within the vasculature, to be directly transported into the brain across the cerebral vasculature by endocytosis, seems to depend on both their size and the nature of the external chemistry and charge of the particle. If they are coated with surfactants such as polysorbate, nanoparticles can more readily cross the BBB (Gulyaev *et al.*, 1999; Schroeder *et al.*, 2010).

3.3.2 Phagocytosis

In general, immune cell transfer across the BBB is restricted and very limited. However, there seems to be a minor but important entry of phagocytes into the brain that then emerges as microglia (Hickey, 1991). Any elevation of generalized oxidative stress associated with excessive levels of systemic inflammation can allow an increased recruitment of monocytes into brain tissue (Drevets *et al.*, 2009). Oxidative stress can also activate microglia to produce inflammatory cytokines (Yang *et al.*, 2011).

3.3.3 Disruption of Blood–Brain Barrier

The normally tight junctions of the cerebral capillaries normally severely limit diffusion of blood constituents into the brain. Nonetheless, particles with diameters <12 nm may be able to pass directly across the BBB (Sarin *et al.*, 2009). However, damage to the BBB can lead to a nonspecific “leakiness” where exudate from the capillaries can directly access nerve tissue. Such increased membrane permeability is known to be caused by a wide range of disorders associated with inflammatory conditions. These include Alzheimer’s

disease (Ryu and McLarnon, 2009), trauma (Preston, Webster, and Small, 2001), multiple sclerosis (Pfeiffer *et al.*, 2011), alcoholism (Abdul Muneer *et al.*, 2012) and reperfusion injury (Abo-Ramadan *et al.*, 2009). Nanoparticles composed of metal oxides are able to cause cerebral edema and disrupt the BBB (Sharma *et al.*, 2010; Trickler *et al.*, 2012).

3.3.4 Neurotoxicity in Absence of Entry of Triggering Agents into the CNS

Deranged Brain Metabolism Secondary to Generalized Systemic Changes

A single intra-tracheal instillation of single walled carbon nanotubes impaired the growth of brain in mice. This effect was greater than any deficit found in other organs (Park *et al.*, 2011). Since there was extensive evidence of systemic inflammation, this effect may generalized inflammation rather than to direct penetrance of these particles (which have a high aspect ratio), into the brain. It is possible for the expression of neurotoxic changes to take place following exposure to particulate matter in the absence of frank entry of material into the brain. Highly asymmetric carbon nanotubes are especially capable of provoking inflammatory responses (Jain *et al.*, 2011; Kim *et al.*, 2011; Murphy *et al.*, 2012) and can induce mesothelioma as effectively as asbestos fibers (Takagi *et al.*, 2008). Despite the unlikelihood of their entering the brain, such materials must also be considered to have significant neurotoxic potential. The issue of whether inhaled particulates can alter brain function without entry into the brain, by affecting systemic stress is certainly valid and has not been considered sufficiently (Valberg, Long, and Hesterberg, 2008).

4 MECHANISMS UNDERLYING PARTICULATE-RELATED NEUROLOGICAL DAMAGE

4.1 General Reactions, Common to many Materials

A postulated common trajectory: Inflammation leading to oxidative stress leading to DNA damage and altered genomic expression (Figure 1).

4.1.1 Inflammatory and Oxidant Basis of Damage to CNS Induced by Particulates

All persistent ultrafine particles have the capacity to act as an inflammatory stimulus. This is true of airborne particles such as silica and asbestos in the lung (Fubini and Hubbard, 2003) and particles finding their way into body cavity such as talc during surgery (Edlich *et al.*, 2001). The harmfulness of these materials seems to be related to the immune responses mounted by the body. These are ineffective as they are unable to clear these non-proteinaceous particles. This can lead to recruitment of more macrophages, leading to a growing and prolonged focus of irritation. It is not surprising then that insoluble nanoparticles within the CNS have repeatedly been reported to lead to evidence of heightened inflammatory and oxidative activity. Such particulates include manganese (Elder *et al.*, 2006), iron (Wang *et al.*, 2011a,b), silica (Choi *et al.*, 2010) and diesel fumes (Win-Shwe and Fujimaki, 2011). The consequences of particulate-related chronic inflammation are known to be involved in oxidative stress and ultimately genetic changes. Once again, the brain appears to be no exception to such sequelae (van Berlo *et al.*, 2010). Evidence of nanoparticle-induced excess damage attributable to oxidant free radicals has been reported (Donaldson *et al.*, 2005; Peters *et al.*, 2006; Wang *et al.*, 2008; Rahman *et al.*, 2009), as well as damage to DNA (Calderon-Garciduenas *et al.*, 2003).

4.1.2 Susceptibility of Dopaminergic Pathways

Nervous tissue has one neurotransmitter, dopamine that is especially vulnerable to oxidative damage (Bondy and Sharman, 2007) and is a critical component of several neural pathways. Damage to this species can have significant adverse behavioral outcomes. There is evidence that these pathways are specifically susceptible to disruption by airborne particulates (Zhang *et al.*, 2011a; Wu *et al.*, 2011; Wu, Ding T, Sun, 2013). The fostering of oxidant free radicals, would account for the unusual susceptibility of dopaminergic systems to these materials.

Behavioral change and their neurophysiological processes are the ultimate test for adverse neurotoxic events and these have both been reported for several metal oxides in nanoparticulate form including Pb (Oszlanczi *et al.*, 2011), (Papp *et al.*,

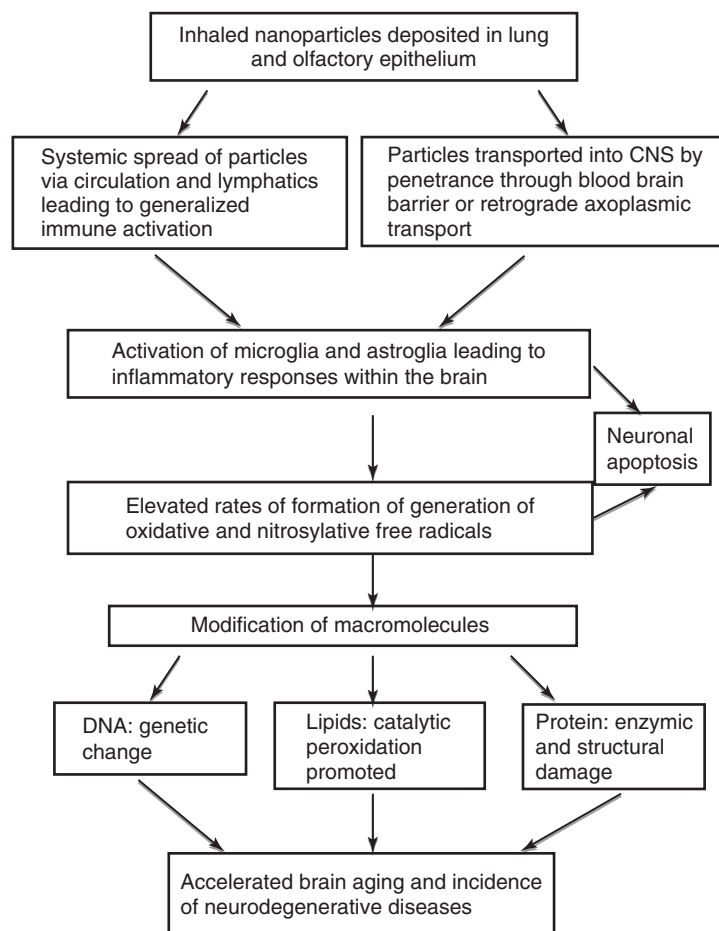


Figure 1. Trajectory by which particulates may promote progression of neurodegenerative disorders.

2012), Ti (Wang *et al.*, 2011a,b), Mn (Oszlanczi *et al.*, 2010, 2011), Al (Zhang *et al.*, 2011a), and Zn (Han *et al.*, 2011). Such behavioral changes are likely to involve malfunctioning dopaminergic circuitry.

4.1.3 The Form and Size of Particles

Large surface area confers distinctive properties on nanoparticles. Both the harmful properties and the potential beneficial applications of nanoparticles, in large part are related to their extraordinary large surface area. This permits the superficial adsorption of a variety of gaseous and liquid materials. Such absorbed chemicals can greatly affect the biological properties of particles. In addition, surfaces of nanoparticles, by attracting reactive chemicals,

can act as sites of catalytic activity accelerating processes that would otherwise be negligibly slow. The large surface area of nanoparticles, which is a strong determinant of their toxicity (Lison *et al.*, 1997), can allow for the adsorption of transition metals on their surfaces. This is able to strongly promote redox fluxes. It is noteworthy that asbestos fibers that have been soaked with deferoxamine in order to remove any superficially bound iron, are much less toxic than the native fibers (Goodglick and Kane, 1990). Similarly, the toxicity of colloidal manganese is greatly enhanced by the presence of trace amounts of iron. Synthetic iron-free nanofibers are toxicologically inert, whereas “Even the smallest iron contamination imparts radical reactivity, and toxicity” (Turci *et al.*, 2011). Free radical production on the surface of chemically inert

colloids or nanoparticles is increased by two orders of magnitude by the presence of trace amounts of transition metals activity (Yang, Guo-Ross, and Bondy, 1999; Bondy and HaMai, 2004; Voinov *et al.*, 2011). Even bimetallic nanoparticles that are homogeneous but are composed of a redox active transition metal together with a more redox inert metal, can promote oxidant events more effectively than either metal alone (Lee and Sedlak, 2008).

Activation of materials by incomplete sequestration of the surface of particles may also apply to low molecular weight organic materials found in conjunction with many airborne particulates. Both mutagenic and oxidant species have been found to be absorbed on nanoparticles derived from combusted diesel fuel (Tahara *et al.*, 1994; Matsumoto, Adachi, and Suzuki, 2005). Even gold particles, which are completely chemically inert, can bind and thus activate oxidants (Sardar *et al.*, 2009). Nanoparticles can thus consist of a relatively inert matrix with a high capacity for binding many direct oxidants and reactive chemicals on its very large surface. Such interactions may greatly enhance the toxicity of nanoparticles (Baeza-Squiban *et al.*, 1999).

In addition to the size and shape of nanoparticles, modification of their surface hydrophilicity can also affect the extent to which they can access brain compartments (Zhang *et al.*, 2011b). Thus, the entry into the brain of particles and the subsequent derangements that may be initiated, are both highly dependent on a range of physical and chemical characteristics. When combined with the heterogeneity of many particulates suspended in gaseous media, this complicates the task of identifying the potential neurotoxic hazards to be expected.

4.2 Mechanisms Specific to Individual Agents

While there seem to be several features common to the mechanism of neurotoxicity of all nanoparticles, the chemical nature of their constituents obviously plays a major role in their mode of action. This more specific toxicity of chemicals is likely to be closely related to the means by which they are neurotoxic when in a form other than fine particles. Thus, metals capable of valence transitions under biological conditions can cause redox flux leading to generation of reactive oxygen species.

Part of the neurotoxicity of particles containing iron, manganese, or copper can be attributed to such free-radical-promoting processes. The extent of leaching and dissolution of metals from particle surfaces can also determine their degree of toxicity. The particulate toxicity of metals known to promote prooxidant conditions due to their affinity for sulfhydryl groups, such as mercury, cadmium and zinc, will persist whether the metals are present as constituents of nanoparticles or in other forms.

5 RELATION TO NEUROLOGICAL DISEASE

5.1 Nanoparticles and the Promotion of Age-Related Neurodegenerative Disorders

Pathological changes have been reported in healthy dogs chronically exposed to heavily polluted air in Mexico City (Calderon-Garciduenas *et al.*, 2003.). These changes included evidence of inflammation, especially in the olfactory bulb and hippocampus. Excess brain levels of metal associated with oil combustion (vanadium and nickel) were found. There was also an acceleration of Alzheimer's-type pathology. The commonalties between the pathology of air pollution and that of Alzheimer's and Parkinson's diseases include early pathology in the olfactory bulb, and pathways with olfactory deficits being one of the earliest findings in both diseases. In addition, exposure to urban air pollution has been shown to cause both neuroinflammation and accumulation of $A\beta_{42}$ (component of $A\beta$ plaques) and α -synuclein (component of Lewy bodies), leading to the suggestion that neurodegenerative diseases can be promoted by particulate air pollution. While these field studies are relatively uncontrolled, both as to the complex number of particulate and gaseous constituents in polluted urban air and the heterogeneous strains of dog used, they are important in that they reflect a "real world" environment to which a large population is exposed. The relevance of such an ill-defined mixture may be furthered, as it is here that crucial synergistic events between components may take place. Diesel exhaust contains over 40 constituents known to be toxic. Some of these are particulate including several heavy metals and a variety of ill-defined carbonaceous materials, whereas others are gaseous such as NO, CO, ozone

and uncombusted hydrocarbons (Hesterberg *et al.*, 2006).

5.2 Combined Hazards can Expose Vulnerability

However, it is obviously needed to enhance such reports by using more controlled laboratory experiments involving the inhalation of atmospheres, where the gaseous and particulate components are well defined. Such artificial designs can be especially revealing when particulate inhalation is combined with a separate stressor such as hyperthermia. A genetically produced knockout mouse lacking an ApoE gene renders this line more liable to Alzheimer's-like pathological progression. Evidence of inflammatory changes has been found in brains of such animals following inhalation of nanoparticles derived from ambient air (Campbell *et al.*, 2009). The presence of disease such as diabetes (Lafuente *et al.*, 2012), influenza (Elder *et al.*, 2004), or hypertension (Elder *et al.*, 2007) can be of value in eliciting particulate neurotoxicity. Such animal models illustrate that there may be sub-populations especially vulnerable to nanoparticulates.

6 SUMMARY

The initiation of inflammatory events within the brain by exogenous ultrafine particles is based on several factors:

1. Although particulate inclusions may be chemically inert, their small size and large surface area can activate glial cells to express immune responses. Microglia and astroglia can be immunocompetent and microglia can be provoked into assuming phagocytic characteristics reminiscent of those of systemic macrophages (Zotova *et al.*, 2011). These events can be further enhanced by infiltration of circulating macrophages into the brain.
2. The extensive aqueous-solid interface of very small particles in biological tissue can act to partially chelate metal ions. In the case of transition metals capable of expressing more than one valence state under physiological conditions (Fe, Cu, and Mn), this loose binding can form an

effective platform for Fenton-like redox cycling activity. These fluxes can result in rapid catalytic generation of reactive oxygen species. In a manner parallel to the ability of asbestos to induce oxidative damage to DNA being dependent on traces of iron, the toxicity of small amounts of transition metals can powerfully synergize with that of nanoparticles.

7 CONCLUSION

In designing studies on the neurotoxicology of nanoparticles, two opposing forces are at play. One requires experimental conditions and the materials used should be well defined and with thorough investigation of a very limited number of variants. The other need is for the complexities of particle exposure in the real world to be recognized. A large range of possible interactions and synergistic events exist between particles and other components of the atmosphere. It is likely that the true hazards of complex mixtures, such as polluted air of diesel fumes, cannot be understood by integration of results derived from findings derived from individual components. There is then a conflict between the need for more mechanistic understanding and the need to identify from a regulatory perspective, the true hazards of ill-defined yet prevalent assortments of materials.

While this issue remains a challenge, some unequivocal facts relating to nanoparticle exposure are unfolding. Nearly all age-related chronic disease involves up-regulation of inflammatory pathways. These are often related to the aging process and include cancer, cardiovascular disease, Alzheimer's disease, Parkinson's disease, arthritis, diabetes, and obesity (Ahmad *et al.*, 2009; Prasad, Sung, and Aggarwal, 2012). Despite this, effective immune function is depressed with aging resulting in increased susceptibility to infection and reduced responses to vaccination (Cannizzo *et al.*, 2011). Thus, immunosenescence involves depression of signal/noise activity leading to inappropriate, often harmful reactions rather than to effective responses. The toxicity of airborne particulates often includes evidence of such heightened inflammation. This is not confined to lung but is broadly systemic (Yokota *et al.*, 2005). A single, short-term inhalation exposure to diesel engine exhaust can trigger gene expression changes in rat brain to an extent

comparable to those observed in the lung (van Berlo *et al.*, 2010). It remains unclear whether the observed effects in the CNS result from direct access of materials to the brain, or whether generalized systemic mediators cause them indirectly. The difficulty in addressing this is that, by the time particulate effects are found in nerve tissue, they are also often evoking more widespread reaction throughout the body.

RELATED ARTICLES

Chapter 3
Chapter 5
Chapter 8
Chapter 12
Chapter 13

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Nanoparticles and Plants: From Toxicity to Activation of Growth

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1 INTRODUCTION

Industrial development of promising nanobiotechnologies has the potential to increase significantly the use of nanomaterials in agriculture, horticulture, plant biotechnology, and the biofuel industry in the near future. The ability of nanoparticles to penetrate plant cells has generated a strong interest in utilizing nanoparticles as smart treatment-delivery systems in plants (Gonzalez-Melendi *et al.*, 2008; Torney *et al.*, 2007). Using bombardment method, Torney *et al.* (2007) proved that gold-capped mesoporous silica nanoparticles (MSNs) penetrated cell walls and delivered DNA to plant cells. Nanocapsules (nano-devices with an internal reservoir) can be used to deliver herbicides to plants (Perez-de-Luque *et al.*, 2006). This delivery method provides better penetration through plant tissues and allows slow and constant release of herbicides (Perez-de-Luque *et al.*, 2006). Silver nanoparticles (Ag NPs) are another promising class of nanomaterials that can be used to fight plant diseases. As previously reported, Ag NPs have strong bactericidal and antifungal effects (Panacek *et al.*, 2006; Lara *et al.*, 2010a, b; Lara *et al.*, 2011). Their effectiveness has been demonstrated in the reduction of plant diseases caused by spore-producing fungal pathogens (Jo, Kim, and Jung, 2009; Krishnaraj *et al.*, 2012) and in the reduction of microbial growth on plant cuttings (Solgi *et al.*, 2009; Liu *et al.*, 2009). However, the extensive use of nanomaterials for new biological

applications can result in their diffusion in air, landfills, and water sources (Oberdorster, 2004; Joner, Hartnik, and Amundsen, 2008; Dietz and Herth, 2011). Nanomaterials can be absorbed by plants via many entry routes and translocated within the plant body (Corredor *et al.*, 2009; Ma *et al.*, 2010; Khodakovskaya *et al.*, 2011). Plants constitute the largest interface between the environment and the biosphere, therefore, the potential toxicity of nanoparticles and their applications remain very serious concerns.

2 UPTAKE AND TRANSLOCATION OF NANOMATERIALS INSIDE PLANTS

Nanomaterials can be subdivided into carbon-based materials (CBMs) and metal-based materials (MBMs) (Rico *et al.*, 2011). CBMs include the fullerene C₇₀, the fullerol (C₆₀(OH)₂₀), carbon nanotubes (CNTs), graphene, and nanohorns, whereas the MBMs include TiO₂, CeO₂, FeO, ZnO, and Al₂O₃ (Rico *et al.*, 2011). The changes in physical and structural properties of significantly small size materials are raising a question about possible routes of uptake and adverse effect of nano-sized materials in plants (Corredor *et al.*, 2009; Ma *et al.*, 2010). The presence of cell wall in plant cells is reducing their permeability to nanoparticles (Eggenberger *et al.*, 2010). However, the ability of single-walled and multi-walled CNTs to penetrate

intact plant cell walls of isolated tobacco cells was demonstrated by two research groups (Liu *et al.*, 2009; Khodakovskaya *et al.*, 2012).

Little is known about subcellular localization of CBM or MBM inside plant cells. It was proposed that nanoparticles escape endocytosis and diffuse into the cytoplasm through direct penetration (Serag *et al.*, 2011). Once inside cells, multi-walled CNTs were found to follow a size-dependent translocation into different cellular structures: vacuoles, nucleus, and plastids contained 30–100 nm multi-walled CNTs, whereas long, multi-walled CNTs (<200 nm) were found in other organelles, such as mitochondria and endoplasmic reticula (Serag *et al.*, 2011).

Nanomaterials can be delivered to whole plants via an aerial or root pathway (Zhang *et al.*, 2012). Absorption, translocation, and accumulation of different nanomaterials through plants have been observed in numerous crops. Airborne nanomaterials are categorized as ultrafine particles with dimensions less than 0.2 μm (Dietz and Herth, 2011). Their size facilitates their entry into leaves through stomata (Birbaum *et al.*, 2010) and into the intracellular fluid through cuticle permeation (Schreiber, 2010). After an aerosol exposure, CeO_2 nanoparticles were deposited on the surface of leaves and shoots of young *Zea mays* plants. These particles were incorporated into plant tissue and were not removed by surface washing (Birbaum *et al.*, 2010). Magnetite nanoparticles (Fe_3O_4) were found inside the epidermal cells of the petiole after spray application to pumpkin stems and in xylem vessels 48 h after injection into the stems of pumpkin plants (Corredor *et al.*, 2009).

Nanomaterials can be easily absorbed by plants germinated or grown in solutions supplemented with nano-sized materials. A study by Larue *et al.* (2012) revealed that the translocation of titanium dioxide nanoparticles in wheat roots to the vascular cylinder was size dependent (Larue *et al.*, 2012). Nanoparticles with diameters smaller than 36 nm were able to cross the parenchyma and the Casparian band to reach the xylem vessels (Larue *et al.*, 2012). The dynamic uptake and distribution of carbon nanoparticles in rice plants exposed to C_{70} (fullerene) and germinated in buffer supplemented with nanoparticles have been described by Lin and Xing (2008). The presence of C_{70} noted in the first generation exposed seeds and leaves as well as in rice leaves of the second generation

(Lin *et al.*, 2009b). Uptake of multi-walled CNTs by tobacco cells (Khodakovskaya *et al.*, 2012) and tomato seedlings (Villagarcia *et al.*, 2012) from agar medium supplemented with CNTs was documented using transmission electron microscopy (TEM) and Raman spectroscopy. A recent study reported that multi-walled CNTs supplied to the soil mix through a watering system are easily absorbed by the roots of tomato plants (Khodakovskaya *et al.*, 2011). Advanced methods of detection, including photothermal/photoacoustic (PT/PA) analysis and Raman spectroscopy, have revealed the ability of soil-located CNTs to enter tomato plant and reach tomato reproductive organs (flowers and fruits) (Khodakovskaya *et al.*, 2013; Khodakovskaya *et al.*, 2011).

These observations raise an essential question about the safety of plants that absorbed significant amounts of nanomaterials. By accumulation of nano-sized particles, plant organs could initiate biomagnification (Holbrook *et al.*, 2008; Rico *et al.*, 2011; Judy, Unrine, and Bertsch, 2011). A comprehensive assessment of the risks associated with toxicity of nano-exposed plant organs (leaves, roots, fruits, and seeds) to animals and humans that consume plants is warranted.

3 RISKS ASSOCIATED WITH POSSIBLE ENTRY OF NANOMATERIALS INTO THE FOOD CHAIN

The toxicity of nano-sized materials has been documented in animal systems (Brasseur *et al.*, 1980; Nelson *et al.*, 1993; Heinrich, 2013). Symptoms of disruption of the cytoskeleton organization of human fibroblasts caused by iron nanoparticles were observed in cell culture studies (Gupta and Gupta, 2005). CNT-induced DNA damage was demonstrated in mouse embryonic stem cells (Zhu *et al.*, 2007), which suggest the possibility of developmental toxicity to humans. Poland *et al.* (2008) demonstrated that CNTs introduced into the abdominal cavity of mice show asbestos-like pathogenicity such as the formation of granulomas (Poland *et al.*, 2008). In another study, researchers demonstrated that multi-walled CNTs migrate to the subpleura and increase the accumulation of pleural mononuclear cells and subpleural fibrosis in mice (Ryman-Rasmussen *et al.*, 2009). The basic mechanisms of the toxicity of nanomaterials

for biological tissues are not yet clear, but it has already been discovered that much depends on the nanoparticles' specific elemental composition, electronic structure, shape, surface coating, state of aggregation, solubility, and interactions with other environmental factors (Yang and Watts, 2005; Nel *et al.*, 2006; Tiede *et al.*, 2008). An increase of lipid membrane peroxidation through the release of reactive oxygen species (ROS) in cells exposed to nanoparticles may play an influential role in the phenomenon of nanotoxicity (Nel *et al.*, 2006). In addition, nanomaterials can affect the genomic level of an organism. For example, CNTs can activate the expression of genes involved in cellular transport, metabolism, and stress signaling in human skin fibroblasts (Ding *et al.*, 2005).

4 TOXICITY OF NANOMATERIALS FOR PLANTS AND SOIL MICROBIAL COMMUNITY

Phytotoxicity of different types of nanomaterials has been widely studied in recent years (Yang and Watts, 2005; Dietz and Herth, 2011; Khodakovskaya *et al.*, 2013). Under certain condition, any nano-sized material can be toxic for plants. The age and type of plants used for nano-toxicological experiments are particularly significant parameters for specific plant response. The level of toxicity will also depend on the major properties of material, including chemical structure (toxic metals), size, shape, aggregation, concentration, functional groups or attachments, method of delivery, and purity of nanomaterial used (Rico *et al.*, 2011). Many nanomaterials contain impurities that could be highly toxic to plants. For instance, synthesized CNTs have been reported to contain nickel, yttrium, cobalt, iron, molybdenum, copper, lead, and chromium (Plata, Gschwend, and Reddy, 2008). Because specific impurities are not identical for experimental studies, the comparison of toxicological data produced by different research groups is extremely difficult.

Numerous plants, including important crops, developed signs of toxicity from exposure to two categories of nano-sized materials: MBM and CBM. Phytotoxicity associated with exposure to nanomaterials was recorded as phenotypic observations of (i) seed germination, (ii) root elongation, and (iii) shoot length and biomass.

4.1 Effects of Nanomaterials on Seed Germination

Seed germination is the key phase in the development of any plant. Under favorable conditions, seeds can germinate and develop into healthy plants. The presence of MBM or CBM in the growth medium affected the rate of germination of seeds of different plant species. For example, zero-valent iron nanoparticles completely inhibited the germination of flax, barley, and ryegrass seeds (El-Temsah and Joner, 2012). A dose-dependent negative effect of chromium oxide nanoparticles on seed germination of *Triticum aestivum* has been described (Vajpayee *et al.*, 2011). Ag NPs decreased seed germination of barley and ryegrass up to 50% (El-Temsah and Joner, 2012). Tomato, cucumber, and maize seed germination was dramatically affected by CeO₂ at concentrations of 500–2000 mg l⁻¹ (López-Moreno *et al.*, 2010). The toxic effect of nanoparticles on seed germination could be due to the permeability of the seed coat to some types of nanomaterials. For instance, Lin and Xing suggest that the germination of the several crop seeds was not greatly affected in one study (Lin and Xing, 2007) because of low or no permeability of the seed coats to five types of nanoparticles.

4.2 Effect of Nanomaterials on the Root System

The root is the organ that is typically in direct contact with the agar or liquid that can be supplemented with nanoparticles. It was shown that nanoparticles (TiO₂) can negatively affect root hydraulic conductivity and water flow through roots of maize (Asli and Neumann, 2009). The harmful effect of MBM on root elongation has been reported in several studies. Application of suspensions of 2000 mg l⁻¹ Zn or ZnO nanoparticles severely damaged the roots of ryegrass (Lin and Xing, 2007). The negative effect of Cu nanoparticles on zucchini (Stampoulis, Sinha, and White, 2009) and wheat (Lee *et al.*, 2008), Al, Zn, and ZnO nanoparticles on corn, lettuce, and ryegrass (Lin and Xing, 2007), and CeO₂ nanoparticles on tomato and alfalfa (López-Moreno *et al.*, 2010) root growth was confirmed. Al₂O₃ nanoparticles inhibited root development of maize, cucumber, carrots, cabbage (Yang and Watts, 2005), and corn (Lin and Xing, 2008). A few studies report

the effects of CBM on root elongation of different crops. For example, functionalized single-walled CNTs ($104\text{--}1750\text{ mg l}^{-1}$) reduced the root length of tomato, lettuce, and zucchini (Canas *et al.*, 2008).

4.3 Effect of Nanomaterials on Biomass Accumulation and Development of Reproductive Organs

Plant growth, biomass accumulation, and the development of reproductive organs are affected by nano-sized materials applied in high doses. For example, the application of single-walled CNTs delayed flowering and decreased the yield of rice plants (Lin *et al.*, 2009a). Exposure to growth medium containing multi-walled CNTs reduced the biomass of zucchini by 38% (Stampoulis, Sinha, and White, 2009). The biomass of zucchini was decreased to 69 and 90% in response to the application of Ag or Cu nanoparticles, respectively (Stampoulis, Sinha, and White, 2009). Negative effects on seedling and shoot growth were observed: (i) in barley and ryegrass after treatment with Ag aqueous suspension (El-Temsah and Joner, 2012), (ii) in mug bean and wheat after growing on agar medium supplemented with Cu nanoparticles (Lee *et al.*, 2008), and (iii) in alfalfa and corn after treatment with CeO_2 nanoparticles (López-Moreno *et al.*, 2010).

In recent years, phytotoxicity of new nanomaterials, such as graphene, was monitored for a number of crop species (Begum, Ikhtiar, and Fugetsu, 2011). Cabbage, tomato, red spinach, and lettuce grown in a solution supplemented with graphene at a concentration higher than 500 mg l^{-1} have exhibited serious defects in development (Begum, Ikhtiar, and Fugetsu, 2011). An increased level of ROS production was reported (Begum, Ikhtiar, and Fugetsu, 2011). Thus, an oxidative stress mechanism could be the key behind the phytotoxicity of graphene.

4.4 Cytotoxic Effect of Nanoparticles

While most studies on toxicity rely on phenotypical observations, cytotoxicity studies on the negative effects of nanoparticles on plants are quite limited. However, investigators have noted that the cytotoxic response of plants to the application of nanomaterials is dependent on the size,

aggregations, and concentrations of the nanoparticles. Kumari, Mukherjee, and Chandrasekaran (2009) reported that Ag NPs led to a decrease in mitosis, a disturbance of metaphase and cell wall disintegration when applied to *Allium cepa* (Kumari, Mukherjee, and Chandrasekaran, 2009). Studies on multi-walled CNTs applied to rice showed that even at low concentration (20 mg l^{-1}), chromatin was condensed inside the cytoplasm and caused cell death (Tan, Lin, and Fugetsu, 2009). Moreover, the plasma membrane detached from the cell wall and led to cell shrinkage (Tan, Lin, and Fugetsu, 2009). According to a report by Shen *et al.* (2010), single-walled CNTs induced programmed cell death in *Arabidopsis* and rice protoplasts (Shen *et al.*, 2010). CNTs caused a dose-dependent adverse cellular response in protoplasts, including plasma membrane deposition, cell aggregation, and chromatin condensation (Shen *et al.*, 2010). The genotoxic potential of CeO_2 and ZnO nanoparticles was demonstrated in soybean seedlings by random amplified polymorphic deoxyribonucleic acid (RAPD) assay (López-Moreno *et al.*, 2010). The genotoxicity of TiO_2 in *Allium cepa* was demonstrated using comet assays and DMA laddering (Ghosh, Bandyopadhyay, and Mukherjee, 2010). More research is needed on the effects of nanomaterials on the expression of genes and proteins involved in plant toxic response.

4.5 Toxicity of Nanomaterials on Soil Microbial Community

The contamination of landfills with nanomaterials will not only affect growing plants but may also impact surrounding soil microorganisms. In terrestrial systems, nanoparticles studies are facing difficulties in assessing the toxicity in the soil matrix, because of the presence of other natural nanoparticles (Shah and Belozero, 2009; Wang *et al.*, 2009). As a result, studies of the toxicity of nanoparticles to soil microbes are very limited. It has been reported that synthetic nanoparticles of Ag, CuO, and ZnO have toxic effects on a beneficial soil microbe *Pseudomonas putida* KT2440 (Gajjar *et al.*, 2009). Reported mechanisms of toxicity on bacteria include membrane disorganization, DNA damage, surface-coating-related photocatalytic oxidation, and ROS production (Ge, Schimel, and Holden, 2012). Other MBM, such as silica, palladium,

gold, and copper nanoparticles, had an insignificant influence on the soil microbial community (Shah and Belozerovala, 2009). The same soil was used to germinate lettuce seeds, and no sign of phytotoxicity was observed (Shah and Belozerovala, 2009). Other studies (Tong *et al.*, 2007; Nyberg, Turco, and Nies, 2008) have reported that the introduction of fullerene (C60) to soil has little impact on the structure and function of the soil microbial community. Recently, the effect of multi-walled CNTs, supplied as regulators of plant growth, on the soil microbial community was studied (Khodakovskaya *et al.*, 2013). Advanced molecular methods revealed that the diversity and richness of microbial communities were not affected by the application of CNTs to the soil used for tomato cultivation. However, the application of CNTs to the soil influenced the relative abundance of each bacterial group in soil samples (Khodakovskaya *et al.*, 2013).

5 NANOMATERIALS AS GROWTH REGULATORS

Interestingly, the same nanomaterials that can be toxic for plants in high doses may play a positive role in the physiological processes in plants in low doses. For example, TiO₂ nanoparticles were able to stimulate growth in spinach plants because of activation of photosynthesis and nitrogen metabolism (Zheng *et al.*, 2005; Klaine *et al.*, 2008). CNTs in low doses are characterized as stimulators of seed germination and plant growth. For example, the increase of root length in ryegrass was observed in response to the application of multi-walled CNTs (Lin and Xing, 2007). Similarly, the root growth of onion and cucumber was increased by exposure to single-walled CNTs (Canas *et al.*, 2008). More recently, both types of CNTs (multi-walled CNTs and single-walled CNTs) increased the germination rate of tomato seeds and enhanced the growth of young tomato plants (Khodakovskaya *et al.*, 2011; Villagarcia *et al.*, 2012). Significant phenotypical changes, such as the increase in the number of flower buds and fruits, in mature tomato plants grown on soil supplemented with multi-walled CNTs were detected (Khodakovskaya *et al.*, 2013). At the cellular level, multi-walled CNTs in a wide range of concentrations (5–500 µg ml⁻¹) were able to stimulate cell growth, a 55–64% increase over

control, of tobacco callus culture (Khodakovskaya *et al.*, 2012).

Characteristics of carbon nanomaterials, such as surface chemistry and type of attachments, play a critical role in the physiological response of plants exposed to such materials. The activation of seed germination and tomato plant growth correlated with the specific type and charge of functional groups on the surface, and the level of agglomeration of CNTs (Villagarcia *et al.*, 2012). Specifically, well-dispersed multi-walled CNTs with negative charged groups on the surface had the strongest effect on the germination and growth of tomato plants (Villagarcia *et al.*, 2012).

Multicomponent nanomaterials must be carefully assessed for physiological response of exposed plants. It was previously reported that single-walled CNTs with attached quantum dots provided excellent opportunities for the simple detection of CNTs inside exposed tomato plants; however, the modification to the CNTs changed the typical response of tomato plants to the application of carbon nanoparticles in low doses (Alimohammadi *et al.*, 2011). The physiological response of exposed tomato plants changed from positive (increase of growth) to negative (obvious symptoms of toxicity) when quantum dots were attached (Alimohammadi *et al.*, 2011). This observation highlights the fact that phytotoxicity of nanoconjugates can result from an attached substance but not the principal nanomaterial.

Investigations focused on understanding the effects of CNTs at the molecular level of plant cells are still very limited. In order to better understand the complex mechanisms of activation in plant physiological process resulting from the application of carbon nanoparticles, total gene expression and selected protein expression were monitored in tomato plants exposed to multi-walled CNTs (Khodakovskaya *et al.*, 2011; Khodakovskaya *et al.*, 2012; Villagarcia *et al.*, 2012). Total transcriptome profiling using Affymetrix Microarray Technology was performed in wild-type tomato and plants exposed to multi-walled CNTs (100 µg ml⁻¹; 200 µg ml⁻¹). A large number of genes (91 genes in leaves and 49 genes in roots) were differently expressed in 10-day-old tomato seedlings exposed to CNTs compared with wild type (Khodakovskaya *et al.*, 2011). A significant number of identified CNTs-regulated genes were involved in plant stress signaling and cellular response. It is important to note that several genes (subtilisin-like endoprotease,

threonine deaminase, and meloidogyne-induced giant cell protein) that were upregulated in response to CNTs can be also activated by biotic stress factors (pathogens or herbivores). Thus, it is possible that plants exposed to the tubular structure of carbon nanomaterial (CNTs) can develop a molecular response that is similar to plant response to pathogens or herbivore attacks. Genomic experiments revealed that expression of genes encoding water channels (aquaporins) was significantly altered in tomato roots (Khodakovskaya *et al.*, 2011) and tobacco cells (Khodakovskaya *et al.*, 2012) grown on medium supplemented with multi-walled CNTs. Aquaporins protein expression was also higher in tobacco cells exposed

to nanomaterial compared to unexposed cells at specific time points during cell incubation (Khodakovskaya *et al.*, 2012). Aquaporins were identified as key components in plant–water relations, root water uptake, seed germination, cell elongation, reproduction, and photosynthesis (Kaldenhoff and Fischer, 2006; Maurel *et al.*, 2008). Aquaporins play an important role in plant development as well (Aharon *et al.*, 2003). The observed enhancement of tobacco cell growth and the stimulation of tomato germination and growth may be due to the activation of symplastic water transport through CNT-related regulation of aquaporins production in seeds, roots, or individual plant cells (Khodakovskaya *et al.*, 2011; Khodakovskaya *et al.*, 2012; Villagarcia

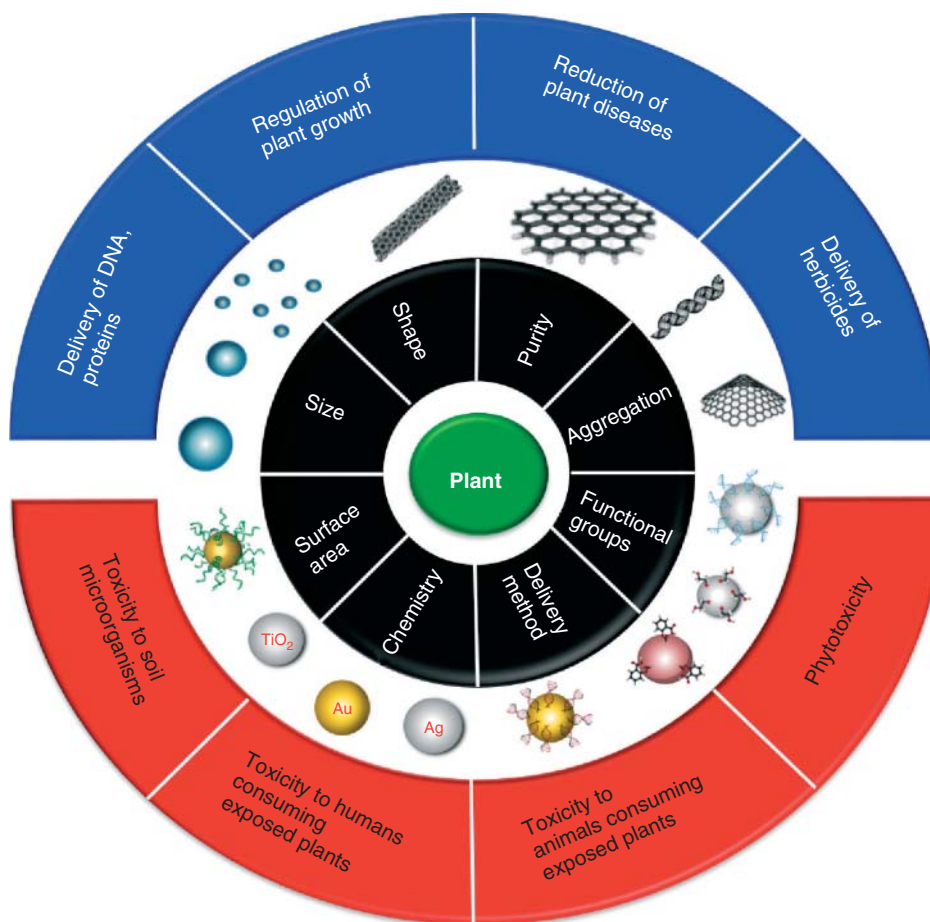


Figure 1. Comprehensive analysis of nanoparticle–plant interaction is critical for assessment of toxic potential of nanomaterials (red case) as well as for development of new technologies (blue case). The different characteristics of nanoparticles (black case) are responsible for response of plant to application of nanomaterial.

et al., 2012; Khodakovskaya *et al.*, 2013). However, microarray analysis (Khodakovskaya *et al.*, 2011) reveals that the effect of CNTs on the plant transcriptome is very complex and cannot be described by the regulation of a single gene. Villagarcia *et al.* (2012) demonstrated with real-time PCR assay that genes regulating cell division (*CycB*) and cell wall extension (*NtLRX1*) are overexpressed in the CNT-exposed tobacco cells that exhibited faster growth than the control cells. Such observations indicate that future comprehensive investigations would be needed to fully understand the molecular mechanisms of positive (activation of growth) effects, as well as the toxicity of nanomaterials in plants.

6 CONCLUSION

Nanomaterials–plant interactions is a new and emerging area of nanobiotechnology. The importance of such studies is related to the development of new applications for plant biology/biotechnology, as well as the assessment of environmental risks from production of nanomaterials in large amounts (Figure 1). Many nanomaterials are described as promising substances for the regulation of plant growth, reduction of plant diseases, and delivery molecules (herbicides, DNA, and proteins) inside plant cells and to specific plant tissues. However, perspectives on such applications will require a clear understanding of the effect of nanomaterials on molecular levels of plant organizations, including transcriptome, proteome, and metabolome. Such studies are quite limited. Accurate assessment of the risks associated with toxicity of nano-exposed plant organs to animals and humans will depend on a number of key factors. First, properties and concentrations of nano-sized materials associated with the toxicity response have to be carefully estimated. Second, ultrasensitive methods for quantitative detection of accumulated nanoparticles in different plant organs must be developed. Currently, methods for detection can visualize nano-sized materials (TEM) inside plant cells (Khodakovskaya *et al.*, 2012) and confirm the presence of nanomaterials inside plant organs (PA/PT, Raman Spectroscopy) (Khodakovskaya *et al.*, 2011; Nedosekin *et al.*, 2011). However, only evaluation of the correlation between the quantity of accumulated nanoparticles inside exposed plants and the toxicity of such

plants for human and animals can clarify associated risks.

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Epigallocatechin-3-gallate (EGCG) in or on Nanoparticles: Enhanced Stability and Bioavailability of EGCG Encapsulated in Nanoparticles or Targeted Delivery of Gold Nanoparticles Coated with EGCG

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1 INTRODUCTION

1.1 Catechins in Tea

Tea made from the evergreen *Camellia sinensis* plant has been consumed for thousands of years and its health-giving attributes have been recorded since ancient times. Nowadays, tea is one of the three most popular beverages in the world (Yang, Lambert, and Sang, 2009). In the past decade, tea has attracted much significant attention in the scientific as well as consumer communities as knowledge related to its health-promoting effects has rapidly increased (Zaveri, 2006). Polyphenolic compounds, particularly the catechins that make up approximately 30% of the dry weight of green tea, are regarded as the principal active ingredients in tea (Graham, 1992). Currently, 12 types of catechins have been identified in green tea (Table 1) and of these epigallocatechin-3-gallate (EGCG) has received particular attention for three reasons: (i) it is the most abundant catechin in green tea, accounting for 65% of the total catechins (Zaveri, 2006); (ii) it has the

highest biological activity compared with other catechins found in tea (Yang, Lambert, and Sang, 2009); and (iii) its interaction with cells is unique, being mediated by the 67-kDa laminin receptor (67LR) on cell surface; as a result, antibodies specific to 67LR are able to efficiently arrest the interaction of EGCG with cells (Tachibana *et al.*, 2004; Shammas *et al.*, 2006; Fujimura *et al.*, 2008; Umeda *et al.*, 2008; Byun *et al.*, 2010; Lee *et al.*, 2010).

1.2 Beneficial Effects and Mechanisms of EGCG

Animal experiments, epidemiological surveys, and human intervention studies have revealed that EGCG possesses numerous health-promoting effects, including but not limited to anti-mutagenic (Santana-Rios *et al.*, 2001), anti-inflammatory (Lin and Lin, 1997; Babu, Si, and Liu, 2012), anticancer (Yang *et al.*, 2001), antiviral (Chen *et al.*, 2012; Calland *et al.*, 2012), antibacterial (Friedman, 2007), and anxiolytic (Vignes, Maurice, and Lante,

Table 1. Catechins in tea.

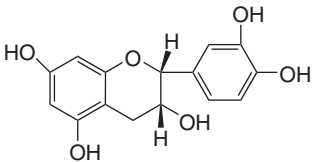
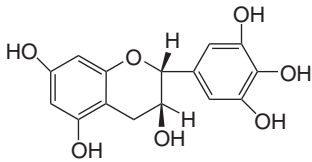
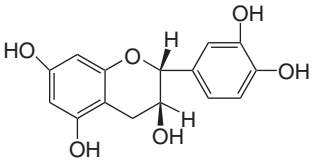
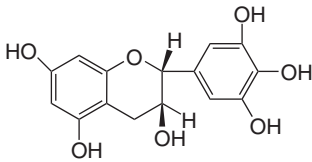
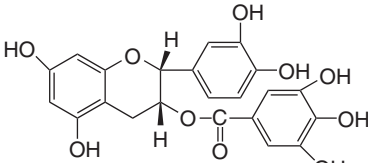
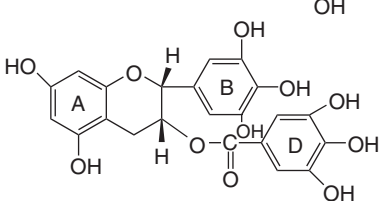
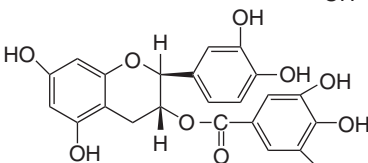
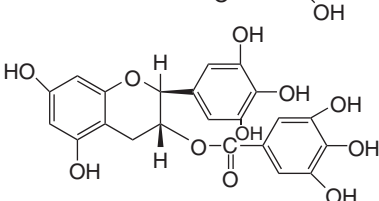
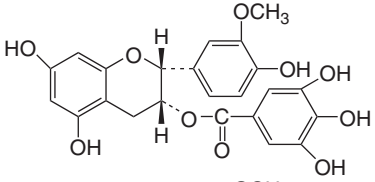
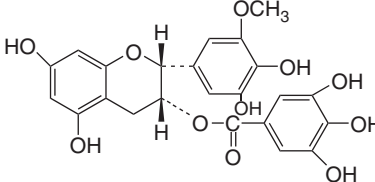
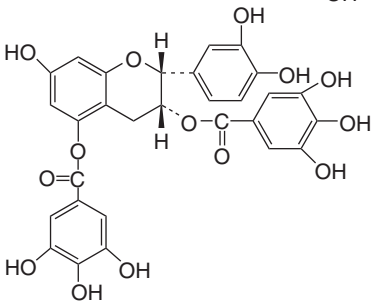
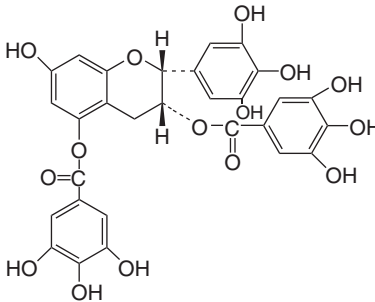
Full name	Abbreviation	Constitutional formula
(-)-Epi-catechin	L-(-)-EC	
(-)-Epi-gallocatechin	L-(-)-EGC	
(+)-Catechin	D-(+)-C	
(+)-Gallocatechin	D-(+)-GC	
(-)-Epi-catechin-3-gallate	L-(-)-ECG	
(-)-Epi-gallocatechin-3-gallate	L-(-)-EGCG	
(-)-Catechin-3-gallate	L-(-)-CG	
(-)-Gallocatechin-3-gallate	L-(-)-GCG	

Table 1. (Continued)

Full name	Abbreviation	Constitutional formula
L-(–)-Epi-catechin-3 (3'-methoxy)-gallate		
L-(–)-Epi-gallocatechin-3... (3'-methoxy)-gallate		
(–)-Epi-catechin-3,5-digallate	L-(–)-EC-DG	
(–)-Epi-gallocatechin-3,5-digallate	L-(–)-EGC-DG	

2006) properties, hypoglycemic, hypolipidemic, and antiobesity actions (Raederstorff *et al.*, 2003; Klaus *et al.*, 2005; Wolfram *et al.*, 2006; Bose *et al.*, 2008), improvement in cognitive learning (Haque, Hashimoto, and Katakura, 2006), prevention of neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease (Rezai-Zadeh, Shytle, and Sun, 2005; Kim *et al.*, 2010), protection of cardiovascular system (Stangl *et al.*, 2007), and bone health promotion (Devine *et al.*, 2007).

The biological functions of EGCG are usually attributed to its antioxidant properties. As shown in Figure 1, at low dose levels, EGCG neutralizes excess and thus harmful reactive oxygen species (ROS) and reactive nitrogen species (RNS) such as superoxide anion, hydroxyl radical, singlet oxygen, peroxy radical, nitric oxide, nitrogen dioxide, and peroxynitrite (Yang, Lambert, and Sang, 2009). The biological mechanism of EGCG at moderate dose levels seems to be somewhat complex. At these dose levels, through auto-oxidation, EGCG incipiently

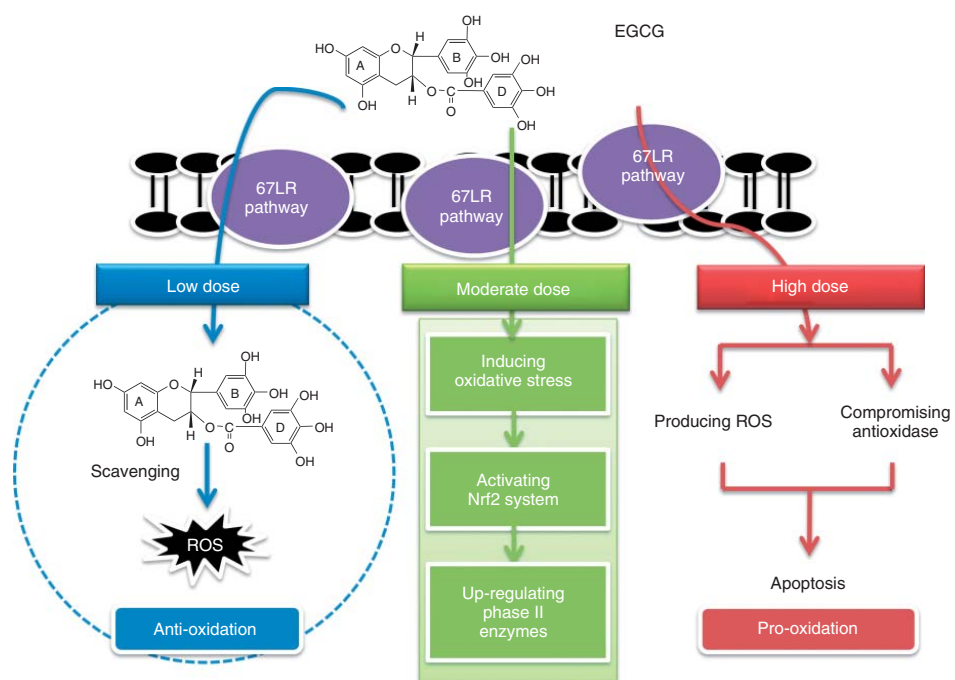


Figure 1. Dose-dependent effects of EGCG on cells.

generates moderate amounts of hydrogen peroxide, which then stimulates the translocation of the nuclear factor erythroid 2-related factor 2 (Nrf2) from cytoplasm into nucleus. Subsequently, Nrf2 together with other elements initiates transcription of a number of antioxidant and detoxifying enzyme genes including glutathione *S*-transferases, quinone oxidoreductase 1, heme oxygenase 1, thioredoxin 1, thioredoxin reductase 1, glutathione peroxidase 2, and uridine diphosphate glucuronyl transferase whose promoters contain antioxidant response element for Nrf2 binding (Brigelius-Flohé *et al.*, 2012; Im *et al.*, 2012; Ma, 2013; Shelton and Jaiswal, 2013). Such an adaptive response to moderate doses of EGCG not only instantly repairs EGCG-initiated redox disturbance but also ultimately confers cells-enhanced antioxidant and detoxifying capacities (Na and Surh, 2007; Chou *et al.*, 2000). At high dose levels, EGCG through auto-oxidation produces large amounts of hydrogen peroxide that cannot be neutralized by the adaptive response mechanism, thereby establishing an intracellular prooxidant environment favoring apoptosis or cell cycle arrest (Li *et al.*, 2010; Wang *et al.*, 2009; Nakagawa *et al.*, 2004). The entry of EGCG into cells is mediated by 67LR that is overexpressed in abnormal cells such as cancer cells (Tachibana *et al.*, 2004; Shammas *et al.*, 2006; Fujimura *et al.*, 2008; Umeda *et al.*, 2008; Byun *et al.*, 2010; Lee *et al.*, 2010). Therefore, EGCG-engendered cytotoxicity acts mainly on abnormal cells, provided that EGCG doses at higher levels are carefully chosen.

Although numerous health-promoting effects of EGCG have been revealed, its applications in disease prophylaxis and treatment are affected by at least two factors: one is its chemical instability and the other is its low bioavailability. We herein summarize achievements in improving the stability and bioavailability of EGCG by encapsulating EGCG in nanoparticles. For more information related to bioavailability of nanomaterials, see Chapter 9. As the entry of EGCG into cells is mediated by 67LR, which is overexpressed on the surface of cancer cells, EGCG may mediate targeted delivery of drugs, provided that EGCG can act as a capping agent on therapeutic payloads, in this regard, we herein recapitulate the innovative finding by employing EGCG as both a capping agent and a targeting agent. For more information related to the influence of nanomaterials on cancer treatment, see Chapter 10.

2 ENHANCED STABILITY AND BIOAVAILABILITY OF EGCG ENCAPSULATED IN NANOPARTICLES

2.1 Stability

As a chemopreventive agent, EGCG can exert its actions by serving as either an antioxidant or a prooxidant, depending on the exposed doses. EGCG consists of a meta-5,7-dihydroxyl-substituted A ring and trihydroxy phenol structures on both B and D rings (Table 1). The polyphenolic structure allows electron delocalization, conferring the ability to quench free radicals (Wiseman, Balentine, and Frei, 1997; Sang *et al.*, 2007). The vicinal trihydroxy structure not only contributes to the antioxidative activity of EGCG but also increases the susceptibility of EGCG to air oxidation under alkaline or neutral pH (Yang, Lambert, and Sang, 2009). Under cell culture conditions or during storage, factors such as pH, temperature, oxygen level, antioxidant level, metal ions, and EGCG concentration are able to affect EGCG's stability, resulting in the formation of several oxidized products such as EGCG quinone, dimer, and dimer quinone (Sang *et al.*, 2007). Therefore, regardless of antioxidative or prooxidative roles in certain types of biological systems, maintaining the stability of native EGCG is crucial for achieving its inherent bioactivities. Li *et al.* (2012) found that the antioxidant activity of EGCG could be efficiently preserved when it was loaded in heat-treated β -lactoglobulin nanoparticles. Wisuitiprot *et al.* (2011) demonstrated that the degradation of catechins extracted from green tea increased in response to the elevated temperature; however, the degradation of the catechins encapsulated in chitosan microparticles was significantly less than that of free catechins. For example, while free EGCG subjected to heat treatment for 24 h showed a degradation ratio of 100%, EGCG encapsulated in chitosan microparticles degraded by only about 50% under the same conditions. Barras *et al.* (2009) reported a more efficient approach for maintaining EGCG stability. In their study, EGCG was loaded in lipidic nanocapsules through a phase inversion process. EGCG in the as-prepared lipidic nanocapsules suspended in water maintained its stability for over 4 weeks without degradation, whereas free EGCG dissolved in water exhibited 100% degradation within 4 hours. Taken together, it is evident that EGCG encapsulation, whether in

the form of β -lactoglobulin nanoparticles, chitosan microparticles, or lipidic nanocapsules, could efficiently preserve its chemical stability and redox potential. Nonetheless, a justifiable concern can be raised as to whether the bioavailability of EGCG encapsulated in nanoparticles is altered and, if indeed it is, is the bioavailability better or worse compared with free EGCG

2.2 EGCG Bioavailability

Accumulated evidence collectively shows that the bioavailability of intact EGCG following oral consumption is poor (Chen *et al.*, 1997; Lambert *et al.*, 2003; Williamson, 2005; Green *et al.*, 2007; Dube, Nicolazzo, and Larson, 2011). In view of the low bioavailability of EGCG, interventions on EGCG transport have shown promising results in increasing its absorption. Coadministration of EGCG with piperine, which inhibits EGCG glucuronidation in the small intestine, results in a significant increase in EGCG levels in mice, and coadministration of EGCG with genistein, which modulates multidrug resistance proteins mediated efflux, enhances plasma exposure of EGCG in mice (Lambert *et al.*, 2004; Lambert *et al.*, 2008). In addition, chemical modification has been employed to increase EGCG bioavailability and bioactivities. Lambert *et al.* (2006) demonstrated that a peracetylated EGCG derivative had a significantly higher bioavailability over free EGCG *in vitro* and *in vivo*. Accordingly, administration of the peracetylated EGCG derivative is more effective than EGCG in preventing the shortening of colon length, formation of aberrant crypt foci, and lymphoid nodules in mouse colon stimulated with dextran sulfate sodium (Chiou *et al.*, 2012), and inhibiting the development, growth, and angiogenesis of experimental endometriosis in mice (Wang *et al.*, 2013). These studies imply that additional efforts to increase EGCG bioavailability are highly warranted.

Recent studies have clearly demonstrated that the bioavailability of EGCG encapsulated in nanoparticles could be significantly improved. In this regard, Siddiqui *et al.* (2009) provided a paradigm. In their study, EGCG was encapsulated in polylactic acid (PLA) and polyethylene glycol (PEG) nanoparticles whose average size was 260 nm. In a prostate cancer model employing athymic nude mice, they found that EGCG in plasma rapidly disappeared within

4 h following intraperitoneal (i.p.) administration of nonencapsulated EGCG at a dose of 1 mg/mouse. Interestingly, EGCG in plasma was still measurable 4 h after i.p. injection of PLA/PEG-encapsulated nano-EGCG at a dose as low as 0.1 mg/mouse, thereby suggesting the pharmacokinetic and pharmacodynamic profiles of PLA/PEG-encapsulated nano-EGCG are altered significantly. Consequently, they found that the two doses with a 10-fold difference could achieve prominent but equivalent therapeutic efficacy in terms of tumor inhibition. Impressively, with this 10-fold dose advantage, PLA/PEG-encapsulated nano-EGCG reduced serum prostate-specific antigen more effectively (from 25 ng ml⁻¹ of the tumor control to 2.6 ng ml⁻¹) than nonencapsulated EGCG (from 25 ng ml⁻¹ of the tumor control to 9.3 ng ml⁻¹). Furthermore, they revealed that such a 10-fold dose advantage could still be observed *in vitro*. In human prostate cancer PC3 cells, in order to achieve 50% growth inhibition, the required concentrations of nonencapsulated EGCG and PLA/PEG-encapsulated nano-EGCG were 43.6 and 3.7 μ M, respectively; to cause apoptosis of the same cell line at a level of 72%, the required concentrations of nonencapsulated EGCG and PLA/PEG-encapsulated nano-EGCG were 40 and 2.7 μ M, respectively. Consistent with this, the 10-fold dose advantage could also be observed in an *in vitro* fibroblast growth factor-induced angiogenesis model (Siddiqui *et al.*, 2009). A similar demonstration was achieved by another group; Singh *et al.* (2011) showed that EGCG and theaflavin, a functional constituent in red tea, encapsulated in poly lactic-co-glycolic acid (PLGA), retained biological effectiveness with over a 20-fold dose advantage compared with free EGCG and theaflavin in exerting anticancer effects in lung carcinoma A549 cells, cervical carcinoma HeLa cells, and acute monocytic leukemia THP-1 cells. At the same dose levels, while free EGCG or theaflavin exerted antioxidant functions, the PLGA-encapsulated nano-EGCG or theaflavin had already begun to initiate prooxidant responses (Alotaibi *et al.*, 2013). It is worth noting that the one order of magnitude dose advantage seen with PLA/PEG- or PLGA-encapsulated nano-EGCG could be reproduced using a different encapsulation method. Lemarié *et al.* (2013) employed SpanTM 60, cholesterol, and SolulanTM C24 to encapsulate EGCG; the resultant vesicles, whose average size was 209 nm, were at least 9-, 9-, and 17-fold more

efficient than free EGCG in causing cell death at a level of 50% in A431 human epidermoid carcinoma cells, B16–F10 mouse melanoma cells, and T98G human glioblastoma cells, respectively.

Certain types of nanoparticles have a tendency to become unstable in the gastric environment. PLA or PLGA nanoparticles are efficient drug delivery systems, but they are prone to hydrolysis in gastric fluid, thereby limiting their use in oral delivery (Siddiqui and Mukhtar, 2010). By introducing PEG, the copolymerization partially overcomes the impact of gastric fluid (Jain *et al.*, 2010); however, it has been noted that EGCG nanoparticles encapsulated in PLA/PEG are still unstable in an acidic environment (Siddiqui *et al.*, 2010). Therefore, to overcome this deficit in oral consumption, several other encapsulation approaches were investigated. Chitosan-encapsulated nano-EGCG is known to be stable in the gastric environment (Dube, Nicolazzo, and Larson, 2010); moreover, EGCG encapsulated in chitosan nanoparticles exhibited a feature of high intestinal permeation as evidenced in a CaCo² cell experiment. The maximum apparent permeation rate (P_{app}) of nonencapsulated EGCG was $0.35 \times 10^{-6} \text{ cm s}^{-1}$, but the maximum P_{app} value of EGCG encapsulated in chitosan nanoparticles was increased to $1.3 \times 10^{-6} \text{ cm s}^{-1}$ (Hu *et al.*, 2012). Dube, Nicolazzo, and Larson (2011) further demonstrated that oral administration of EGCG encapsulated in chitosan nanoparticles significantly enhanced the plasma exposure of EGCG in mice. In addition, Smith *et al.* (2010) found that oral absorption of EGCG into the systemic circulation was greatly improved when it was encapsulated in lipidic nanoparticles. The EGCG plasma concentration in rats treated with EGCG encapsulated in lipidic nanoparticles at 10 min after oral gavage was 599 ng ml^{-1} . In comparison, the EGCG plasma concentration in rats treated with free EGCG was only 117 ng ml^{-1} . The pharmacokinetic parameters, including maximum plasma concentration, area under the curve, and relative bioavailability of EGCG encapsulated in lipidic nanoparticles in rats increased by approximately 6-, 2.5-, and 2.5-fold relative to free EGCG, respectively. All these studies clearly demonstrate that EGCG encapsulation, whether in the form of PLA/PEG (Siddiqui *et al.*, 2009), PLGA (Alotaibi *et al.*, 2013), SpanTM 60/cholesterol/SolulanTM C24 (Lemarié *et al.*, 2013), chitosan (Dube, Nicolazzo, and Larson, 2011; Hu *et al.*, 2012), or lipidic nanoparticles

(Smith *et al.*, 2010), increases its bioavailability and bioactivities *in vitro* and *in vivo*. Considering that EGCG encapsulation in nanoparticles increases its chemical stability, EGCG encapsulation in nanoparticles is able to simultaneously overcome its two major deficits, chemical instability and low bioavailability. The enhanced bioavailability of EGCG encapsulated in nanoparticles can be attributed in part to the increased chemical stability; however, the 67LR-mediated interaction of free EGCG with cells may also play a role. The abundance of 67LR on cell surface may be a limiting factor for the interaction of free EGCG, whereas EGCG molecules encapsulated in nanoparticles can enter cell by endocytosis pathway without being affected by 67LR (Figure 2).

EGCG encapsulation not only improves its stability and bioavailability/bioactivities, but also can be further employed to fulfill targeted delivery of EGCG to cancer cells in order to enhance its chemopreventive and therapeutic potentials. For instance, EGCG encapsulated in PLGA and PEG nanoparticles whose surfaces were assembled with small organic molecule with high binding affinity to prostate-specific membrane antigen (PSMA) exhibited an enhanced cell killing capacity in PSMA-expressing prostate cancer cells compared to the nontargeted EGCG nanoparticles (Sanna *et al.*, 2011). Similarly, as transferrin receptors are overexpressed on cancer cells (Daniels *et al.*, 2006; Calzolari *et al.*, 2007), EGCG encapsulated in transferrin-bearing vesicles exhibited increased cell killing ability compared to EGCG encapsulated in control vesicles, by 1.9-fold in A431 human epidermoid carcinoma cells, 2.7-fold in B16–F10 mouse melanoma cells, and 4-fold in T98G human glioblastoma cells (Lemarié *et al.*, 2013). In mice bearing tumors, EGCG encapsulated in transferrin-bearing vesicles administered intravenously resulted in rapid tumor regression within the first 24 h; however, this phenomenon could not be observed in mice subjected to intravenous treatments with EGCG encapsulated in control vesicles or free EGCG. Most tumors in mice treated with EGCG encapsulated in transferrin-bearing vesicles continued to regress over the following month, leading to complete disappearance in 40% of the mice by day 30. In contrast, all tumors in mice treated with either EGCG encapsulated in nontargeted vesicles or free EGCG continued to grow (Lemarié *et al.*, 2013).

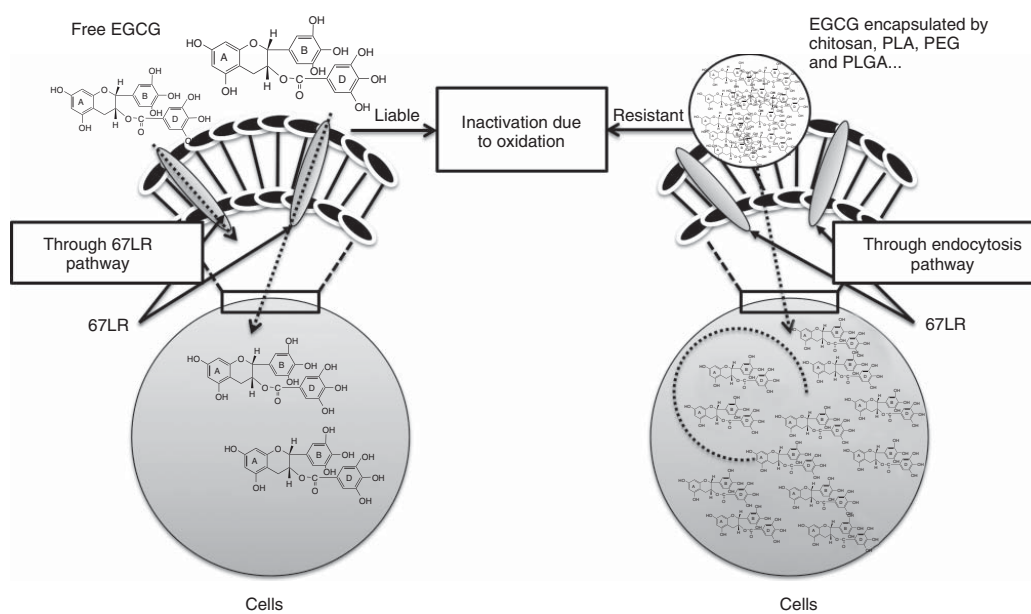


Figure 2. Stability and bioavailability of free EGCG or EGCG encapsulated in nanoparticles.

3 TARGETED DELIVERY OF RADIOACTIVE GOLD NANOPARTICLES TO TUMORS VIA EGCG COATING

^{198}Au is an artificial radioisotope with a short half-life of 2.7 days, a maximum beta-emitting energy of 0.97 MeV, and a maximal tissue penetration depth of 3.8 mm (Tuovinen and Kettunen, 1957; Duggan and Weijer, 1961). Radioactive colloidal gold, with many unique physicochemical properties, has been used to treat various diseases such as malignant effusions induced by chest fluid, ovarian cancer, abdominal cancer, and arthritis (Millar and Macdonald, 1956; Grahame, Ramsey, and Scott, 1970); superficial malignant bladder lesions (Tuovinen and Kettunen, 1957); malignant melanoma (Jantet *et al.*, 1964); craniopharyngiomas (Kodama, Matsukado, and Uemura, 1981); and idiopathic myelofibrosis (Turner, Gummerman, and Boggs, 1985). Nevertheless, considering that efficient therapy with radioactive colloidal gold largely relies on administration route and dose, it is inevitable to confront the drawbacks commonly seen in radiation therapy and chemotherapy, namely limited drug retention in tumor sites and prominent drug penetration into normal tissues (Boisselier and Astruc, 2009). Side effects including paraneoplastic calcification, urological complications, diffuse fibrosis (Deeths and Stanley, 1976), hepatic cirrhosis, and neoplasms derived from reticuloendothelial cells (Upton, Furth, and Burnett, 1956) have been noted during clinical application of radioactive colloidal gold. In the early 1950s, Rubin H. Flocks began to treat prostate cancer patients with radioactive colloidal gold. Over the next 20 years, more than 1500 patients with locally advanced prostate cancer received radioactive colloidal gold treatment under the supervision of Flocks. Intratumoral injection of radioactive colloidal gold in these patients showed statistically significant improvements in short- and long-term survival compared to other contemporary treatments (Rosevear *et al.*, 2011). Very recently, Shukla *et al.* (2012) developed a far more efficient approach to prostate cancer therapy by injecting radioactive colloidal gold into tumors with the aid of a compound found in tea leaves, EGCG.

3.1 EGCG Acts as an Efficient Reducing Agent and an Excellent Stabilizer for Fabricating Radioactive Gold Nanoparticles (AuNPs)

Normally, gold nanoparticles (AuNPs) are prepared using a reducing agent to transform sodium tetrachloroaurate (NaAuCl_4) into Au. The redox potential of EGCG is 420 mV, whereas that of $\text{AuCl}_4^-/\text{Au}$ is 990 mV; therefore, EGCG and tetrachloroaurate constitute a thermodynamically favorable redox couple. EGCG indeed is able to reduce AuCl_4^- to AuNPs (Shukla *et al.*, 2012). Not only that, the as-prepared AuNPs were also simultaneously coated with excess EGCG in the redox system. Transmission electron microscopy showed that the average core size of the EGCG-coated AuNPs was 40–55 nm, whereas dynamic light scattering revealed that the average size of hydrodynamic EGCG-coated AuNPs was 120–130 nm, thereby indicating that the EGCG coating over the AuNPs was thick enough, as high as twice the core size of the AuNPs. The zeta potential of EGCG-coated AuNPs was -37.7 mV, which suggests that EGCG provides robust shielding around AuNPs to prevent against the aggregation of AuNPs. Indeed, EGCG-coated AuNPs in a closed vial could maintain their stability without any noticeable aggregation over 12 months at room temperature. *In vitro* stability experiments with EGCG-coated AuNPs were further carried out in various biological fluids, including 10% NaCl, 0.2 M histidine, 0.5% human serum albumin, and 0.5% bovine serum albumin at pH 7 and 9 to predict the stability of EGCG-coated AuNPs under *in vivo* conditions. Alterations in surface plasmon resonance and plasmon band width were used to estimate stability. The trivial shifts in surface plasmon resonance and plasmon band width under these treatments imply that EGCG-coated AuNPs are stable *in vivo*. Indeed, intact AuNPs without aggregation could evidently be seen using transmission electron microscopy in prostate cancer cells subjected to treatment with EGCG-coated AuNPs. All these lines of evidence collectively demonstrate that EGCG is an excellent stabilizer for AuNPs (Nune *et al.*, 2009; Shukla *et al.*, 2012). It is worth noting that under 100% green chemistry conditions, without the involvement of any harmful chemicals, EGCG is able to act as both an efficient reducing agent to transform

NaAuCl₄ into AuNPs and simultaneously an excellent capping agent to prevent against aggregation of the AuNPs.

3.2 EGCG Not Only Acts as a Reducing Agent and a Stabilizer, It Also Plays a Pivotal Role as a Targeting Agent of Radioactive AuNPs for High Gold Retention in Tumors and Low Gold Leakage into Normal Tissues

¹⁹⁸Au distribution after a single dose of EGCG-coated ¹⁹⁸AuNPs was investigated in an animal model using severely compromised immune-deficient (SCID) mice bearing tumor xenografts of human PC-3 prostate cancer (Shukla *et al.*, 2012). When EGCG-coated ¹⁹⁸AuNPs were injected into already established tumors, the ¹⁹⁸Au beta-emitting radioactivity that was retained in the tumors versus leaked into normal tissues always exhibited a sharp contrast at any time point over the next 24 h, being prominent in the tumors while hardly detectable in the liver and stomach and negligible in blood and multiple other organs including heart, lung, kidney, bone, muscle, brain, pancreas, and bladder, thereby suggesting that a high therapeutic efficacy without perceived toxicity could be achieved. In a therapeutic study using the same animal model and the same drug administration mode, while tumor volume rapidly increased in both the saline and the EGCG treatment groups, tumor growth in the EGCG-coated ¹⁹⁸AuNP-treated mice remained static throughout the whole experimental period. The end-of-study ¹⁹⁸Au distribution on day 42 again showed that ¹⁹⁸Au was mainly present in tumors, which possessed 37.4% of the injected radioactivity, whereas liver had only 2.5% of the injected radioactivity and other organs had negligible radioactivity. On the other hand, blood parameters such as white cells, red cells, lymphocytes, and hemoglobin showed no clinical signs of toxicity. Taken together, the optimal ¹⁹⁸Au distribution using EGCG-coated ¹⁹⁸AuNPs indeed led to a high therapeutic efficacy with a feature of being well tolerated.

There were no significant differences between the EGCG and the saline treatments in terms of tumor growth in the aforementioned therapeutic experiment. EGCG alone thus did not have any therapeutic effect at a dose level equivalent to the EGCG-coated ¹⁹⁸AuNPs. Therefore, it could be envisioned that

the remarkable therapeutic effect engendered by the EGCG-coated ¹⁹⁸AuNPs appears to be mainly attributable to ¹⁹⁸AuNPs, and the function of EGCG in this situation is not immediately indicated. However, it should be emphasized that radioactive gold retention in tumors subjected to EGCG-coated ¹⁹⁸AuNPs or size-equivalent ¹⁹⁸AuNPs capped with Gum Arabic, a glycoprotein known as a *stabilizer of AuNPs* (Nune *et al.*, 2009), was greatly different. At 24-h postdrug injection, in tumors treated with EGCG-coated ¹⁹⁸AuNPs, 1 g of the tissue could retain 200% of the injected radioactivity, whereas, in tumors treated with Gum Arabic-coated ¹⁹⁸AuNPs, 1 g of tissue had only 75% of the injected radioactivity (Shukla *et al.*, 2012). Such a contrast implies that EGCG may provide a targeting effect on cellular uptake of AuNPs. EGCG, but not Gum Arabic, has been known to bind 67LR with excellent specificity and selectivity. Antibodies specific to 67LR are able to effectively arrest the entry of EGCG into cells (Tachibana *et al.*, 2004). Shukla *et al.* (2012) found that treating prostate cancer cells with an antibody specific to 67LR was able to substantially reduce intracellular Au retention by 85%, thereby convincingly demonstrating that EGCG-coated AuNPs are delivered into tumors mainly via the interaction of EGCG and 67LR. It has been documented that 67LR is overexpressed in many types of cancer cells, including prostate cancer cells (Tachibana *et al.*, 2004; Shamma *et al.*, 2006; Fujimura *et al.*, 2008; Umeda *et al.*, 2008; Byun *et al.*, 2010; Lee *et al.*, 2010; Shukla *et al.*, 2012), especially in those with aggressive and metastatic characteristics (Shukla *et al.*, 2012). Therefore, once EGCG-coated AuNPs are injected into tumors, AuNPs are introduced into cancer cells by EGCG via the large quantities of 67LR on the surfaces of the cancer cells, leading to high gold retention in tumors and low gold leakage into normal organs. The EGCG-coated AuNPs paradigm, referred to as a *Trojan horse* approach for targeted delivery of therapeutically radioactive AuNPs, could serve as a basis for designing other therapeutic payloads. In this regard, recently, we have found that elemental selenium nanoparticles could also be coated by EGCG. Selenium compounds and elemental selenium nanoparticles have powerful cytotoxic capacities, provided that they are selectively delivered into tumors (Spallholz, 1994; Zhang *et al.*, 2001; Valdiglesias *et al.*, 2010). The therapeutic potential of EGCG-coated

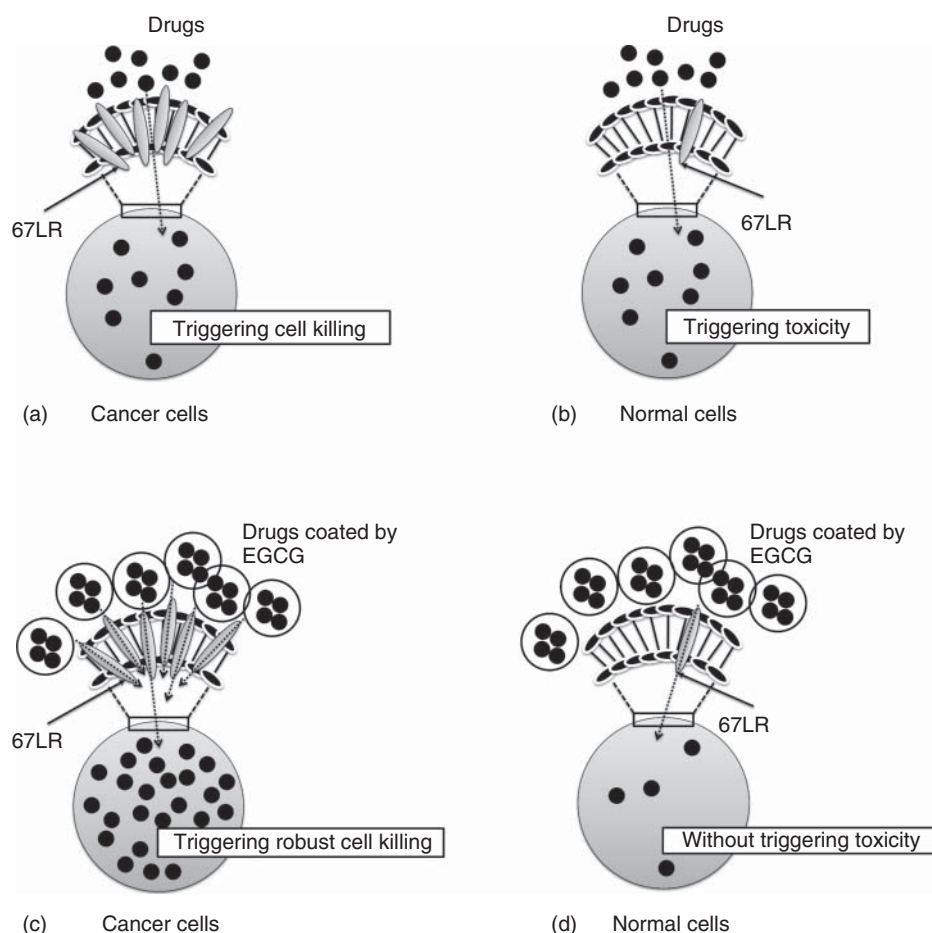


Figure 3. EGCG-mediated drug delivery. Drugs enter (a) cancer cells and (b) normal cells without selectivity. Drugs coated with EGCG enter (c) cancer cells and (d) normal cells with selectivity via the uneven distribution of 67LR.

selenium nanoparticles in an intratumoral injection modality is currently under evaluation in our laboratory.

Overall, EGCG, as an FDA-approved phytochemical for application in humans, plays three roles in fabricating radioactive AuNPs: as a reducing agent, a stabilizer, and, more importantly, a targeting agent. EGCG-functionalized radioactive AuNPs, which were generated under a facile and green route, resulted in potent therapeutic efficacy without perceived toxicity because of the specific affinity of EGCG toward the 67LR that is overexpressed on tumor cells. Accordingly, it could be conceived that EGCG-functionalized other therapeutic payloads would also achieve a high therapeutic efficacy (Figure 3).

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Part Two

Nanomedicine

Factors Affecting the Oral Bioavailability of Nanomaterials

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1 CURRENT HUMAN EXPOSURE

The current use of nanomaterials in food, food packaging, and personal care products (PCPs) increases every year, making both direct and incidental oral exposures a probability rather than a possibility. Most certainly, you are already being exposed to nanoparticles in your food on a daily basis. Do you chew gum? Use nondairy coffee creamer? Drink nonfat milk? Eat any nonperishable food items? If so, current assessments estimate that you eat approximately 40 mg per day of nanosized particles, consisting mostly of TiO₂ and SiO₂ (Powell *et al.*, 2010; Lomer *et al.*, 2004). According to the Woodrow Wilson International Center for Scholars, there were 1000 consumer products containing nanoparticles available in 2009, with a projected market value for nano-related products to reach \$3.1 trillion by 2015 (Powell *et al.*, 2010).

1.1 Direct Food Additives

Direct food additives, used for adjusting the color and texture of food products, are the major source of food-borne exposure to nanomaterials. Silica has been used as an anticaking and thickening agent for several decades and can be found commonly in dry food products. Some examples are listed in Table 1. Some silica-based additives are advertised as nano-silica (e.g., Aerosil 200F and

380F), whereas others such as additive E551 do not specify a size range, but have been found to be 33% nanosized, by weight (Dekker *et al.*, 2011). Of the estimated 40 mg of nanomaterials that we consume each day, 88% is composed of silica additives.

Titanium dioxide is also widely used as a food additive for white coloring and can be found primarily in candies, chewing gum, and toothpaste. Examples are shown in Table 2. While the presence of titanium in chewing gum may not seem like a concern because it is generally not consumed, one group found that between 90 and 95% of the total titanium in a piece of gum was removed and would presumably be swallowed after chewing for 10 min (Chen *et al.*, 2012a). While the total quantity of titanium in most of the gum brands tested was <7 mg per serving, some candies have been shown to have up to 100 mg of titanium per serving (Chen *et al.*, 2012a; Weir *et al.*, 2012). The size of the titanium particles in chewing gums was investigated as well, and between 60 and 90% of the total titanium in a piece of gum, by weight, was found to have a particle size <200 nm (Chen *et al.*, 2012a). Food-grade TiO₂, known as *additive E171*, was reported to have an average particle size <110 nm, with 36% by weight, being <100 nm in diameter (Weir *et al.*, 2012). Titanium dioxide is also widely used in sunscreens and cosmetics because of its UV-absorptive properties and may lead to incidental oral exposures, particularly in children.

Table 1. Example foods containing E551 silica additive: nano-silica content and estimated daily exposures.

Products containing silica additive E551	% of total silica in nano-form (50–200 nm)	Estimated daily exposure (mg)
Instant asparagus soup	33	4.2
Coffee creamer	19	33
Lasagna sauce mix	5	1.7
Burrito seasoning mix	5	0.8
Minced meat seasoning mix	7	0.3
Roasted vegetable rub	12	0.3

On the basis of data from Dekker *et al.* (2011).

Table 2. Titanium content ($\mu\text{g Ti mg}^{-1}$ product) in candy, gum, toothpastes, and other sweets.

Contains: $1 < X < 10 \mu\text{g Ti mg}^{-1}$ product	Contains: $0.5 < X < 1 \mu\text{g Ti mg}^{-1}$ product	Contains: $0.1 < X < 0.5 \mu\text{g Ti mg}^{-1}$ product	Contains: $0.01 < X < 0.1 \mu\text{g Ti mg}^{-1}$ product
<i>Gum</i>	<i>Gum</i>	<i>Gum</i>	
Mentos freshmint	Trident white peppermint	Dentyne fire spicy cinnamon	
Eclipse spearmint	Dentyne ice peppermint		
<i>Candy/sweets</i>	<i>Candy/sweets</i>	<i>Candy/sweets</i>	<i>Candy/sweets</i>
Dickinson's coconut curd	Albertson's vanilla pudding	Albertson's mini marshmallows	Vanilla milkshake
Hostess powdered donette	Betty crocker whipped cream frosting		poptarts
Good and plenty	M&Ms Peanut		Mentos mints
Kool aid blue raspberry M&Ms	Jello banana cream pudding		
	Kool aid lemonade		
	Mother's oatmeal iced cookies		
<i>Toothpaste</i>			
Aquafresh			
Colgate			
Crest cool mint			
Crest mint strip			
Sensodyne			

On the basis of data from Weir *et al.* (2012).

While some foods and PCPs list silica and titanium as ingredients, the current regulations in many countries do not require manufacturers to disclose the quantity or even the presence of all food additives or PCP components (Lomer, Thompson, and Powell, 2002). One group investigated several brands of PCPs (e.g., toothpaste, sunscreen, and deodorant) for titanium content, finding that all products tested had detectable levels of titanium, whereas only 11 of the 32 were labeled properly (Weir *et al.*, 2012). Techniques to measure particles in a complicated matrix such as food have been developed but are currently very time consuming and impractical for high-throughput analysis.

1.2 Food Packaging and Other Uses

Nanoparticles with antibacterial properties are being used in food packaging materials to increase the shelf life of many perishable food items. Silver nanoparticles (AgNPs) were found to be the most widely used nanomaterial in food products during a 2011 survey, a large majority of these applications being food packaging (Bouwmeester *et al.*, 2009; Fröhlich and Roblegg, 2012). Silver particles can be grown on absorbent cellulose pads, used to absorb blood and juices from meat after packaging, and may prevent bacterial growth and their associated unpleasant odors (Fernandez, Picouet, and Lloret,

2010). Silver particles used in medical applications (e.g., wound dressings) have been found to migrate out of the materials in which they are embedded, suggesting that silver particles used in food applications may lead to oral exposure following particle migration from packaging materials (Maqsood, AlSalhi, and Siddiqui, 2010; Fernandez, Picouet, and Lloret, 2010). In developing countries, silver has reportedly been used to decontaminate drinking water and has been suggested as an agent to replace the use of some antibiotics in aquaculture (Duncan, 2011; Vaseeharan, Ramasamy, and Chen, 2010).

Magnesium oxide and zinc oxide are also being used in food packaging materials for preservation and as biosensors for contaminants and pathogens (Bouwmeester *et al.*, 2009; Card *et al.*, 2011; Gerloff *et al.*, 2011). The industry for nano-related pathogen sensing in food packaging has been projected to reach \$7.3 billion by the year 2014 and has the potential to greatly increase quality control of certain foods (Duncan, 2011). Nanomaterials also have extensive use in agriculture as pesticides, fertilizers, and vaccine delivery in livestock (Duncan, 2011). While the potential uses of nanomaterials in the food industry are endless, allowing increased quality control and shelf life of foods, custom food designing, and so on, it is essential that risk analysis and management keep up with these changes and advancements.

1.3 Ingestion of Inhaled Particles

Inhalation is the most extensive route of exposure to small particles that humans experience and has been studied for several decades as part of both environmental and occupational health and safety. While inhalation of particles leads largely to pulmonary exposure, a fraction of inhalants are ultimately ingested. Ingestion of inhaled material is due to the mucocilliary action of the respiratory tract, designed to prevent large particulates from reaching the lungs. Particles $<10\text{ }\mu\text{m}$ are capable of entering the upper respiratory tract; however, only particles $<2.5\text{ }\mu\text{m}$ in diameter are capable of being deposited in the lungs (Hoet, Bröske-Hohlfeld, and Salata, 2004). Inhaled particles larger than $2.5\text{ }\mu\text{m}$ will eventually be moved up the bronchioles and trachea to the epiglottis where they will be ingested, leading to oral exposure. The materials we are exposed to in this way are extremely diverse, including diesel exhaust

components, mining products (minerals, metals, coal, etc.), agricultural products (animal dander, cotton, etc.), and tobacco smoke components, just to name a few. Owing to the size exclusion for lung deposition, the particle sizes being ingested following inhalation will be very large ($>2.5\text{ }\mu\text{m}$). However, the behavior of these materials in the gastrointestinal tract (GIT) may result in particle breakdown and dissolution that is fundamentally different than what we know about pulmonary exposure and should be investigated separately.

1.4 Nanopharmaceuticals

The pharmaceutical industry has been investigating the potential for nano-formulated oral medications for several years. A list of some of the currently available formulations can be found in Table 3. Oral delivery of medications and vaccines is noninvasive and cost efficient, with high patient compliance compared to alternative routes. However, in order to achieve successful systemic distribution, medications must be protected from degradation in the stomach, achieve transport through the GIT protective mucus layer, and, finally, succeed in cellular uptake at the epithelial surface. All of these barriers to GI absorption are important considerations for drug delivery and will also mediate the uptake of particles following incidental and food-borne exposures.

2 BARRIERS TO GIT PARTICLE UPTAKE AND IMPORTANT DETERMINING FACTORS

In order to achieve systemic exposure, particles must overcome the three main barriers to uptake, outlined earlier and shown in Figure 1. Owing to the current extensive use of nanoparticles in food and pharmaceuticals, it is important that we understand the implications of consuming these materials and their characteristics (e.g., particle size, agglomeration behavior, surface charge, and surface chemistry) that contribute to oral bioavailability.

2.1 Surviving the Stomach

Particles entering the stomach are likely to undergo changes because of the harsh high salt and low

Table 3. Nano-formulated pharmaceuticals currently available.

Some selected nanomedicine products currently on the market					
Product	Company	Drug	Formulation	Route of administration	application
Doxil	Sequus pharmaceutical	Doxorubicin	Liposome	Intravenous injection	Kaposi sarcoma in AIDS
Amphocil	Sequus pharmaceutical	Amphotericin B	Lipocomplex	IV infusion	Serious fungal infections
Ambisome	NeXstar pharmaceutical	Amphotericin B	Liposome	IV infusion	Serious fungal infections
Dauno Xome	NeXstar pharmaceutical (Boulder, Colorado)	Daunorubicin citrate	Liposome	IV	Kaposi sarcoma in AIDS
Abelcet	The liposome company (Princeton, New Jersey)	Amphotericin B	Lipid complex	IV infusion	Serious fungal infections
Rapamune	Wyeth/Elan (Madison, New Jersey)	Sirolimus	Nanocrystal particles	Oral	Immunosuppressant in kidney transplant patients
Emend	Merck/Elan (Whitehouse Station, New Jersey)	Aprepitant, MK869	Nanocrystal particles	Oral	For chemotherapy patient to delayed nausea and vomiting
TriCor	Abbott (Abbott Park, Illinois)	Fenofibrate	Nanocrystal particles	Oral	Primary hypercholesterolemia mixed lipidemia, hypertriglyceridemia
Megace ES	PAR pharmaceutical (WoodChiff Lake, New Jersey)	Megaestrol acetate	Nanocrystal particles	Oral	Treatment of anorexia, cachexia, or an unexplained significant weight loss in patients with a diagnosis of AIDS
Abraxane	American biosciences (Blauvelt, New York)	Paclitaxel	Albumin-bound nanoparticles	IV injection	Metastatic breast cancer
Elestrin	BioSante (Lincolnshire Illinois)	Estradiol	Calcium phosphate-based nanoparticles	Transdermal	Treatment of moderate-to-severe vasomotor symptoms (hot flashes) in menopausal women

Bawarski, WE, Chidlowsky, E, Bharali, DJ, Mousa, SA. (2008). Reproduced with permission from Elsevier.

pH environment. Figure 2 shows agglomeration, dissolution, and protein interactions. All of these changes are expected to affect particle uptake and biological effects in the GIT. When particles enter the stomach, available ions (e.g., H^+ , Cl^- , and Na^+) will be attracted to the charged surface, resulting in a collapse of the particle's charged double layer that is responsible for particle–particle repulsion. Without a strong repulsion between particles, agglomeration will quickly dominate, increasing the effective

particle size within the GIT lumen and at absorptive surfaces. Agglomeration in the stomach is an important consideration for size-dependent oral bioavailability that very few studies have addressed. However, several studies using polystyrene and latex have shown that primary particle size can greatly influence the extent and location of uptake and distribution (Hoet, Bröske-Hohlfeld, and Salata, 2004; Card *et al.*, 2011; Jani, Florence, and McCarthy, 1992; Fröhlich and Roblegg, 2012). Unlike metal

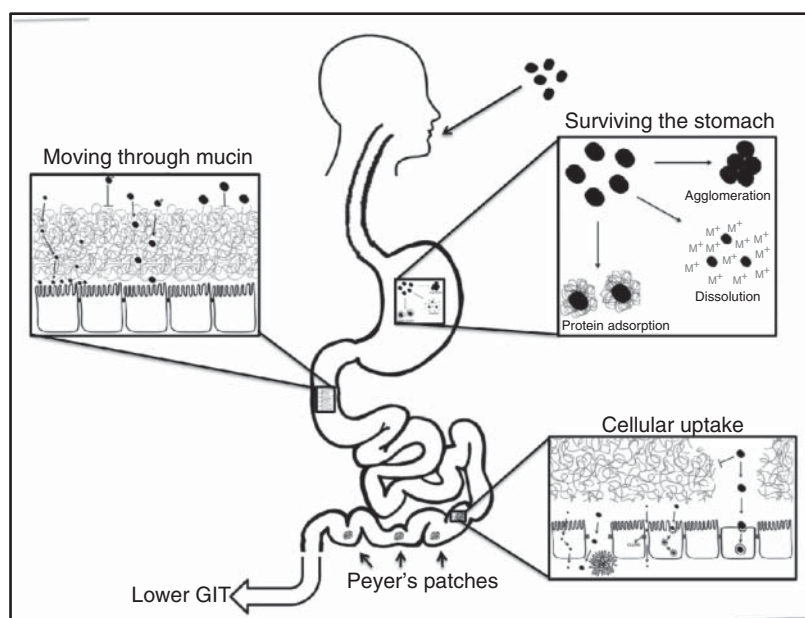


Figure 1. Overview for barriers to gastrointestinal absorption of small particles.

particles, in which the solution stability is very sensitive to pH changes, polystyrene and latex do not experience extensive aggregation in the gastric and intestinal environment and can provide unbiased information about particle size and uptake. Certain polymers, PEG for example, have also been shown to prevent agglomeration and can be used as tools to eliminate this factor from size-dependent investigations of particle uptake (Tobío *et al.*, 2000).

In addition to agglomeration, dissolution should always be considered for metal particles in the stomach. Dissolution will not only decrease particle size but also produce free metal ions, both of which will ultimately increase GIT uptake and systemic distribution. Silver particle dissolution has been investigated most extensively. Studies have shown that particle dissolution increases in parallel with temperature and dissolved oxygen content and that a negative correlation exists between particle dissolution and pH (Kittler *et al.*, 2010; Liu and Hurt, 2010). Along with material, particle size should also be considered as an important factor for dissolution kinetics. One of the special characteristics of nanosized particles is their high surface area-to-mass ratio and is critical in determining the dissolution rate of a particle, with smaller particles dissolving more quickly.

Dissolution and interactions in the stomach are very important to consider for pharmaceutical applications as well. Many medications are unstable in the stomach, degrading in the low pH environment; therefore, nano-formulated medications must exit the stomach before capsule degradation and drug release. Chitosan, for example, is a naturally abundant polysaccharide that is being used and investigated extensively for drug encapsulation due to its biocompatibility. Depending on the synthesis method, chitosan may or may not be resistant to low pH degradation, and care must be taken to ensure that these medications will survive the first barrier to GI absorption (Islam *et al.*, 2012).

2.2 Transiting the Mucin Layer

Present throughout the entire length of the GIT, a thick mucus layer serves several purposes to help maintain the health and integrity of the system. Mucin provides protection for the stomach epithelial membrane against the acidic luminal environment and limits uptake of bacteria in the small intestine and colon to promote local and global immune health. Goblet cells are responsible for maintaining the mucin layer and facilitating constant recycling of the mucin every 4–6 h (Chen *et al.*, 2011; Crater

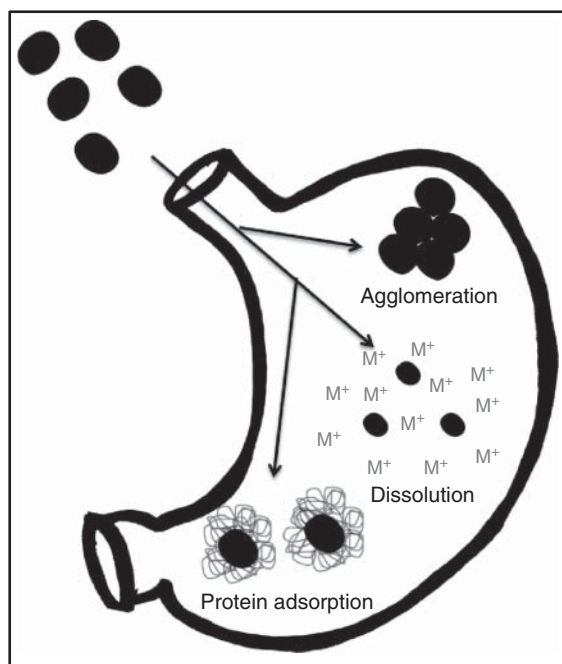


Figure 2. Nanoparticle interactions in the gastric environment: agglomeration, dissolution, and protein corona formation. The principal interactions that take place when particles enter the stomach are shown. The high salt, low pH, and protein-rich environment will lead to agglomeration, dissolution, and protein corona formation.

and Carrier, 2010). Owing to this high turnover rate, it is essential for particles to transit the entire layer in this time frame to achieve cellular uptake. The molecular components that comprise mucin result in a negatively charged layer with a size exclusion of <1000 Da. These inherent aspects make small and neutral or positively charged particles the most efficient at mucin translocation (Crater and Carrier, 2010, Fröhlich and Roblegg, 2012) as depicted in Figure 3. In the rat colon, this layer can be 30–50 μm , increasing in thickness from proximal to distal GIT location and is largely stagnant (Szentkuri, 1997). Despite the apparent size exclusion, Szentkuri has found that 14- and 415-nm latex particles were able to transit the entire rat colon mucin layer in 2 and 30 min, respectively. The largest particles tested, 1- μm latex particles, were unable to transit fast enough to achieve epithelial adhesion during the 4- to 6-h recycling window (Szentkuri, 1997). Chitosan has also demonstrated the ability to transit the GIT mucus layer because of inherent

mucoadhesive properties (Chen *et al.*, 2011; Diab *et al.*, 2012).

Factors affecting mucin health and integrity may influence oral bioavailability and should be considered when assessing exposure to small particles. For example, rats receiving total parenteral nutrition (TPN) have decreased maintenance of the mucus layer, resulting in increased uptake of 3- μm polystyrene beads following oral gavage (Khan *et al.*, 1997; Iiboshi *et al.*, 1994). In addition, diesel exhaust exposure can result in a fivefold increase in mucus permeability, making uptake of larger particles possible due to decreased mucin transit time (Fröhlich and Roblegg, 2012). Mucin effects should always be considered, especially for manufactured particle solutions containing residual surfactants or other complicated matrices, which may be capable of altering mucin maintenance and stability.

2.3 Cellular Transport

Following mucin translocation, particles approach another barrier to systemic exposure: cellular uptake at the epithelial layer (Figure 4). The three dominant cell types in the GIT are goblet cells, enterocytes, and M-cells. Goblet cells, as previously mentioned, maintain the protective mucus layer and are not heavily involved in nutrient or particle absorption. Enterocytes, the most abundant cell type, are responsible for absorbing the nutrients from food through a variety of mechanisms, both passive and active, and are good candidates for uptake of small particles. M-cells are a functional unit of the immune centers of the GIT and are designed to sample the intestinal environment, having been shown repeatedly to absorb very large particles.

2.3.1 Energy-Dependent Uptake

Several groups have demonstrated energy-dependent uptake of nanoparticles via enterocytes using *in vitro* systems. Clathrin-mediated endocytosis has been demonstrated for TiO_2 , SiO_2 , and silver particles (Figure 4a) (Fröhlich and Roblegg, 2012). While it is generally accepted that only particles with a diameter <150 nm are candidates for receptor- or ligand-mediated uptake, much of the available literature demonstrates that surface chemistry is the dominating factor determining mechanism of uptake for energy-dependent pathways (Rieux

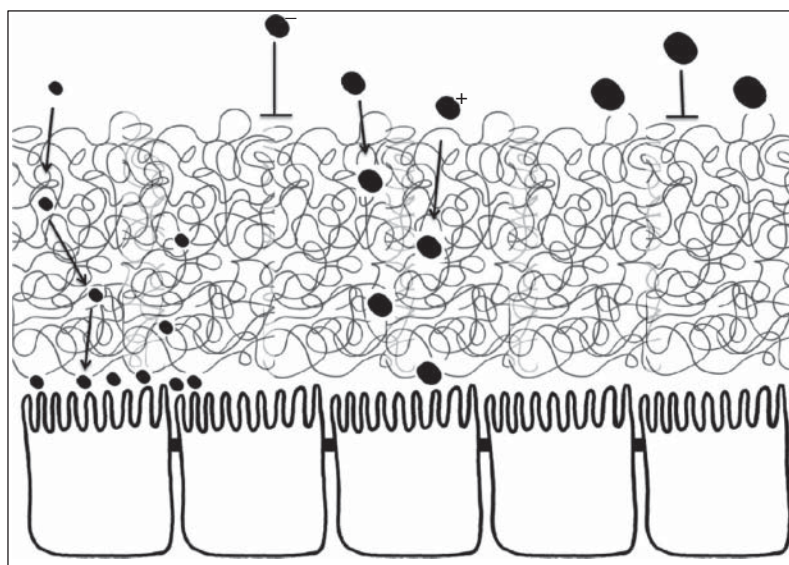


Figure 3. Mucin barrier to uptake: size and charge effects. Small particles move the quickest, accumulating at the cell surface. Intermediate-sized particles are able to translocate through the layer but are much slower and may be inhibited by surface charge. Negatively charged particles are excluded, while neutral and positively charged particles are able to move through mucin. Large particles are unable to move and are excluded from cellular uptake by mucin-coated cells.

et al., 2006). A thorough investigation of 5.5-nm polyethylene glycol (PEG)-coated gold particles in an enterocyte cell culture model has indicated caveolar-mediated endocytosis as the major mechanism of particle uptake (Lin *et al.*, 2012). However, this group found that by using a different surface coating, the mechanism of uptake can be shifted to clathrin-mediated uptake without changing the size, shape, or surface charge of the particles. Other surface coatings, such as tomato lectin, vitamin B₁₂, and wheat germ agglutinin, have demonstrated the ability to increase uptake through cell recognition and receptor-mediated uptake (Chen *et al.*, 2011).

2.3.2 Passive Diffusion

While particle size is less important for determining route of energy-dependent uptake, it is a very critical factor for passive diffusion. This pathway has been demonstrated for various particle types and can occur via three main mechanisms. Lipophilic particles are able to pass directly through the phospholipid bilayer of cells (Figure 4b), with a size exclusion of 700 Da (Chen *et al.*, 2011). Paracellular transport is also a possibility for small lipophilic particles, through the tight junctions (TJs)

of adjacent cells. The standard size exclusion for this pathway is very small, <300 Da, but particles such as chitosan have shown the ability to interrupt TJ integrity by causing the redistribution of essential TJ proteins, known as *claudins* (CLDNs), creating an opening up to 20 nm in width (shown in Figure 4c) (Chen *et al.*, 2012b). TiO₂ particles are also suspected of achieving paracellular transport through unknown mechanisms, being able to cross a Caco-2 cell monolayer without disruption of TJs (Fröhlich and Roblegg, 2012). It is possible that the TiO₂ particles tested were able to achieve passive uptake through cell turnover, an event in which a large opening of several micrometers is briefly available for particle translocation during cell death (Figure 4d). This mechanism of uptake was shown to be the dominant form of systemic exposure to 5- and 10-nm gold particles in mice and has been observed using electron microscopy (Hillyer and Albretch, 2001). It is important to note that these studies performed with gold and titanium do not investigate *in vivo* agglomeration behavior. Therefore, the primary particle sizes reported are not necessarily indicative of the actual particle sizes that were absorbed by the cells in these experiments.

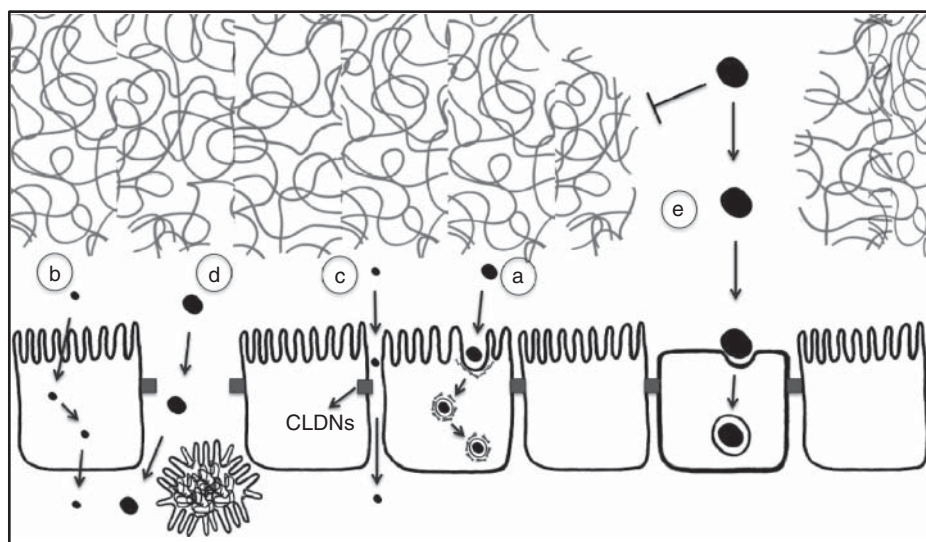


Figure 4. Passive and active mechanisms of particle uptake. Several possible mechanisms of cellular uptake are illustrated for enterocytes and M-cells: (a) clathrin-mediated endocytosis, demonstrated for SiO_2 , TiO_2 , and Ag particles; (b) passive diffusion through the phospholipid bilayer, possible for small/lipophilic particles; (c) paracellular transport, through chitosan-like interactions with CLDN tight junction proteins; (d) passive uptake through cell turnover; and (e) immune-mediated uptake of large particles due to a lack of the mucin barrier.

2.3.3 Immune-Mediated Uptake

Peyer's patches (PPs) are found in the lower part of the small intestine and have an epithelial layer consisting of M-cells, specialized immune cells responsible for monitoring the intestinal environment. Unlike other cells of the GIT, M-cells have no brush border and, more importantly, no mucus layer covering their surface (Figure 4e). By eliminating this barrier to uptake, M-cells are capable of absorbing much larger particles, up to several micrometers. Nanoparticle uptake by M-cells has been shown to be five times greater than by enterocytes, although their intestinal abundance is much lower (Chen *et al.*, 2011). Several studies using fluorescent polystyrene and latex particles have illustrated size-dependent absorption and fate of particles through immune-mediated uptake.

After oral administration in rats, 2- μm polystyrene particles were able to enter PPs and translocate to the mesenteric lymph nodes (MLNs), whereas 9- μm particles remained in PPs and were unable to move on to the MLNs (Florence *et al.*, 1995). Another group investigated the fate and transport of smaller fluorescent polystyrene particles (300 nm to 1 μm) in rats, finding that all particle sizes were taken into PPs and translocated to the MLNs, eventually being

transported to the spleen. The smaller particles were then able to redistribute to other organs, leaving the 1- μm particles sequestered in the spleen (Jani, Florence, and McCarthy, 1992). These studies show clearly that M-cell-mediated uptake and downstream fate and transport are heavily size dependent and that M-cells are able to take up larger particles than the sizes that are available for uptake in other, mucus-coated areas of the GIT.

Proteins adsorbed to a particle surface have also been shown to affect immune-mediated uptake. Immediately on entering a protein-rich solution, particles form a protein corona that will vary based on particle size, shape, and material (Deng *et al.*, 2009; Wasdo *et al.*, 2008). Several groups have investigated specific protein coatings and have found effects on the location and extent of particle uptake. M-cells were found to absorb 20 times more IgA-coated latex particles compared to BSA-coated (bovine serum albumin) particles (Porta *et al.*, 1992). Another group has shown a similar pattern using protein-coated polystyrene particles, demonstrating differential uptake into M-cells depending on specific protein coat. IgG-coated particles were absorbed most extensively, followed by BSA-coated particles and particles coated with bovine growth hormone were the least well absorbed (Smith *et al.*,

1995). Protein coating on particles can also influence the location of uptake; tomato lectin coating on 500-nm polystyrene particles shifted the preferred site of uptake to enterocytes rather than M-cells, despite being too large for traditional routes of enterocyte absorption (Hussain, Jani, and Florence, 1997).

3 ORAL BIOAVAILABILITY AND SYSTEMIC DISTRIBUTION

Focusing on barriers to gastrointestinal absorption and particle characteristics that influence these barriers does not fully explain the behavior of nanomaterials in the GIT. Despite showing that surface charge, protein coating, particle size, and so on can affect particle uptake in a cell culture model or select organs, the larger picture for oral bioavailability is much more complex with several dozen factors to consider. *In vivo* investigations that examine several tissues can give us much more information about oral bioavailability and distribution patterns that cannot be inferred from studies with a smaller scope.

Chronic oral exposure to silver has been widely examined in rats. As dissolution is an important consideration for silver particles, these studies often include a soluble silver-dosing group (silver nitrate (AgNO_3) or silver acetate (AgAc)) for comparison of uptake and distribution. One study, looking at 59-nm uncoated and 49-nm PVP-coated AgNPs and AgNO_3 found similar distribution patterns for all three dosing groups, with liver and spleen having the highest detected levels of silver. Silver was also detected in the testis, kidneys, brain, and lungs of animals from all three groups (Zande *et al.*, 2012). Overall, absorption was lower for AgNP-dosed animals compared to AgNO_3 , suggesting that AgNPs remained at least partially intact following oral administration. Another study, investigating 14 nm AgNPs and AgAc reported comparable findings, with the overall distribution pattern being similar between the two groups and a higher bioavailability for animals dosed with soluble silver (AgAc). Liver and kidney were reported to have the highest level of detected silver followed by lung, muscle, and brain tissue (spleen was not analyzed in this study). During the 28-day oral exposure, feces were collected for a single 24-h period, showing that 63% ($\pm 23\%$) and 49% ($\pm 21\%$) of the daily dose for AgNP and

AgAc, respectively, were excreted by this route. Urinary excretion was shown to be $<0.1\%$ for either group based on the daily dose (Loeschner *et al.*, 2011). A third study investigated 56 nm AgNPs at multiple concentrations in both male and female rats. This group found detectable levels of silver in all tissues tested (testis, kidney, liver, brain, lung, and blood) with higher concentrations in the liver and kidneys of female versus male rats (Kim *et al.*, 2010). This group also reported accumulation of silver in kidney and lung tissue that was higher than the proportional increase in dose, suggesting possible dose-dependent oral bioavailability trends that have not been investigated extensively.

Oral bioavailability of zinc and copper presents a unique situation because of a more strict homeostatic regulation for GI absorption of these metals. Baek *et al.* (2012) investigated 20- and 70-nm ZnO particles, finding a peak in zinc plasma levels at 24 h after oral dosing in rats. They also found that liver, lung, and kidney accumulated the most zinc after dosing and that liver and lung experienced significant redistribution during the first week post-exposure, with peak zinc levels at 1–2 days and 6 h, respectively. TEM investigation failed to locate any solid zinc particles, suggesting that GIT dissolution was very significant for these particles. Their data also suggest that dose can affect absorption, distribution, and retention, with absorption and retention both increasing with dose. Increases in particle size were also reported to cause an increase in retention time (Baek *et al.*, 2012). An investigation into the toxicity and bioavailability of copper particles clearly demonstrates the importance of particle size and dissolution for oral exposures. Chen *et al.* (2006) investigated 17- μm and 23.5-nm copper particles, as well as an ionic copper solution. This group reported that the LD50 for the microparticles was over 5000 mg kg^{-1} , designated as nontoxic, whereas the nano-copper and ionic copper had LD50s of 413 and 100 mg kg^{-1} , respectively (Chen *et al.*, 2006). These data, in addition to tissue analysis, suggest that dissolution plays a very important role in the oral bioavailability and toxicity of copper particles.

Unlike other metal particles, gold is not susceptible to gastrointestinal dissolution and can be used as a tool to eliminate this factor for oral bioavailability. Schleh *et al.* (2012) investigated the influence of particle size and surface charge on oral bioavailability using TPPMS (mono-sulfonated triphenylphosphine) coated 1.4, 5, 18, 80, and 200 and 2.8 nm

positively and negatively charged gold particles. The bioavailability of all particles was <0.5% of the administered dose, with more than 99.5% of the dose being found in the GIT and feces. Despite having low bioavailability, significant differences were observed for both surface charge and particle size. The 2.8-nm negatively charged particles (2.8 nm⁻ Au) had significantly higher uptake in all tissues compared to the positively charged 2.8 nm particles (2.8 nm⁺ Au), with one notable exception; no gold was detected in the brain of animals dosed with 2.8 nm⁻ Au, whereas the level of gold in 2.8 nm⁺ Au-dosed animals was above the limits of detection (Schleh *et al.*, 2012). Size-dependent uptake was observed for most tissues, with 1.4 nm particles having the highest overall absorption (0.37% in all combined tissues) and the highest level in the blood, urine, kidneys, and carcass. Interestingly however, the 18 nm particles were absorbed the most into the brain and heart and were absorbed to a larger extent in the blood and carcass compared to the smaller 5 nm particles (Schleh *et al.*, 2012). These deviations from size-dependent uptake could possibly be due to small differences in particle solutions. The 1.4 and 18 nm particles were synthesized with TPPMS, whereas the 5, 80, and 200 nm particles were synthesized using citrate and then ligand exchanged to TPPMS (Schleh *et al.*, 2012). Despite having similar surface chemistry and charge at dosing, it is possible that residual chemicals from synthesis were able to influence absorption and distribution.

Small differences in dosing solutions such as this illustrate how critical particle characterization can be. Did these particle solutions behave differently in the GIT due to differences that were not detected using TEM and zeta potential analysis? The need for particle characterization standards is discussed in more detail below but can be considered a reoccurring issue for study analysis and affects the extent to which a given study contributes to the state of the science and our understanding of this topic.

4 SPECIAL POPULATION-COMPROMISED INTESTINAL HEALTH

When considering the oral bioavailability of nanomaterials and the implications for food safety and the use of nanosized food additives, it is important to consider a special population for this particular

exposure. Crohn's disease (CD) and ulcerative colitis (UC) affect approximately 0.201 and 0.238% of people in the United States, respectively, and is much more prevalent in developed countries compared to under developed or developing areas (Kappelman *et al.*, 2007; Danese and Fiocchi, 2011; Lomer, Thompson, and Powell, 2002). Animal studies have shown us that one of the major mechanisms of particle uptake is through enterocyte turnover. Owing to an increased cell turnover rate in these diseases, people with colonic inflammation are likely to have higher bioavailability and distribution of orally exposed particles compared to humans with a healthy colon. For example, radioactive gold particles were detected in the liver of patients with inflamed colon tissue compared to no detection in control patients after oral administration (Hussain, Jaitley, and Florence, 2001). Studies done in rats have also suggested that particle uptake may be higher in a chemically induced colitis model, showing that 100 nm and 1- μ m polystyrene particles had a higher rate of adhesion to inflamed colon tissue compared to colon tissue in control animals (Lamprecht, Schäfer, and Lehr, 2001).

Several characteristics of these disorders are expected to increase the oral bioavailability of small particles. Depletion of the protective mucus layer, reduction of TJ integrity, and dysregulation of intestinal lumen pH are all common aspects of CD and UC (Hunter *et al.*, 2012). Mucus removal in a ligated segment of rat colon resulted in a sixfold increase in the absorption of 3- μ m latex particles from the lumen in an *ex vivo* experiment (Khan *et al.*, 1999). Decreased TJ regulation would allow the paracellular transport of particles up to 20 nm in size, without special chitosan-like particle characteristics. Furthermore, unlike intestinal lumen pH in healthy individuals (pH range from 6 to 7), diseased individuals can have a pH range anywhere from 2.7 to 5.5, usually centered on the lower end from pH 2 to 3. This makes particle dissolution possible not only during the average 2 h of gastric exposure but for much of the GI transit time.

Not only are people with CD and UC expected to absorb an increased amount of particles from oral exposure, these particles may be partially responsible for their symptoms. A small pilot study of nine patients with CD found that adhering to a 4-month low-particle diet helped to alleviate some of their disease symptoms (Lomer *et al.*, 2004). The trend for these diseases to be more prevalent in developed

countries also supports the idea that processed foods, containing the most nano-titanium and silica, may influence disease development. Furthermore, a survey of CD and UC patients found that this population has a higher than average intake of processed foods (Lomer, Thompson, and Powell, 2002). Experimental evidence also exists to support this theory; one group found that oral administration of silver particles in rats induces irregular goblet cell activity and alters mucin profiles, reflective of the molecular mucin changes seen in Crohn's patients (Jeong *et al.*, 2010). None of these studies provide conclusive evidence for a cause/effect relationship between intake of small particles in food and the development of CD and UC, but it is something that should be investigated further.

5 METHODS AND TECHNIQUES

As with any scientific inquiry, several factors must be considered when designing a nanotoxicology study. Several of the techniques being used to understand nanomaterial behavior and interaction with biological systems are new and developing, often being employed before their advantages and pitfalls are fully evaluated. There are benefits and shortcomings to any technique available, and understanding these aspects of a study design is crucial in executing a valuable experiment. When choosing the techniques to be used in a nanoparticle study, the specific material being studied will be influential in decision-making.

As discussed earlier, working with polystyrene and latex particles has certain advantages regarding agglomeration issues. In addition, these particles can be easily made to have fluorescent properties, making their detection quite easy. Fluorescent particles are very appealing for cell culture experiments because of the ease of using techniques such as flow cytometry. Using fluorescent particles can also allow gross examination of tissues for presence/absence assessment of oral bioavailability. However, like all situations, there are some pitfalls that researchers should be aware of for these applications. When assessing cell uptake of fluorescent particles using flow cytometry, care must be taken to distinguish between particles that are cell bound versus cell internalized. Using another technique, confocal microscopy, for example, in conjunction with

flow cytometry can help prevent making incorrect assumptions about cell uptake of particles. In addition, when using fluorescent particles, researchers must ensure that the fluorescent tag being detected is stable in the given biological environment and that tag-particle dissociation is not occurring.

Using metal nanoparticles opens several other doors for detection and quantitation techniques. Several methods are currently available to quantitatively and qualitatively assess oral bioavailability of metallic particles, all of which have advantages and disadvantages. Instrumental neutron activation analysis (INAA) and inductively coupled plasma mass spectrometry (ICP-MS) are both techniques that can simultaneously quantify several metals. INAA can be used in very complex systems without destructive sample preparation, unlike acid digestion that is required for ICP-MS. On the other hand, ICP-MS is a much more rapid analytical technique and can analyze many more elements than INAA. Unfortunately, both of these techniques are unable to answer one very important question that is often discussed in oral bioavailability studies of metallic nanomaterials: the particle versus ion dilemma. Owing to the role that dissolution plays following gastric exposure, it is critical to know whether metals being detected in tissues are in the form of a particle or ions. There are two techniques that are able to answer this question, both of which have benefits and limitations. Under different operating parameters, traditional ICP-MS can be transformed into single-particle inductively coupled plasma mass spectrometry (SP ICP-MS), allowing the detection and sizing of individual particles in tissue and food matrices. This method can provide a size distribution of particles found in tissues and can analyze complicated matrices without extensive sample preparation. However, unlike traditional ICP-MS, single-particle mode can only qualitatively detect one metal at a time and the current sensitivity of available instruments limits the size of particles than can be detected, varying based on material density. Electron microscopy can also be used to answer the particle versus ion dilemma, giving much more information about specific cell and subcellular location of particles. However, this method can be expensive and requires extensive sample preparation and technical support. A more comprehensive review of methods for nanotoxicology studies can be found in Chapter 1.

6 LIMITATIONS AND CHARACTERIZATION NEEDS

Several organizations are currently monitoring the use of nanomaterials in food, as well as developing methods for their detection and quantitation: the Nanotechnology Project of the Woodrow Wilson International Center for Scholars, The Global New Products Database of Mintel, The Nanotechnology Product Directory, and the NanoLyse and NanoRelease projects (Bouwmeester *et al.*, 2009). Both the NanoLyse and NanoRelease projects, as well as other groups, have emphasized that adequate particle characterization is essential for assessing oral bioavailability of any nanomaterial (Bouwmeester *et al.*, 2010; Powers *et al.*, 2006). Owing to the complex and harsh environment of the GIT, specifically the stomach, material characterization must be performed in such a way that will allow us to accurately predict the *in vivo* particle characteristics. Many studies correlate particle characteristics of the “as-supplied” or “as-dosed” material to bioavailability and downstream effects, lacking any understanding of how these characteristics may have changed after entering the system.

Performing complete particle characterization for all nanomaterial studies would greatly increase the ability to assess toxicity and biological reactions, elucidating the specific particle characteristics dictating these responses. Unfortunately, this is to some extent, an unreasonable expectation for several reasons, including cost, access to instrumentation, and availability of material. Many characterization techniques are costly to perform and, in addition, may use a large amount of expensive stock materials. Ideally, an exact list of characterization requirements should be supplied and implemented in all studies, yet this list would likely vary based on material and biological end point (Bouwmeester *et al.*, 2010; Oberdörster *et al.*, 2005). Some of the most important characteristics to assess for any study include primary particle size, surface charge and chemistry, agglomeration behavior, impurities and UV, thermal, and pH persistence, and stability. Several methods are available for characterizing these particle features and their particular advantages and pitfalls have been reviewed extensively elsewhere (Bouwmeester *et al.*, 2010; Powers *et al.*, 2006; Calzolari *et al.*, 2012). One of the over-arching themes of these characterization reviews is that multiple techniques should ideally be used to study

any particle characteristic. In addition, it is widely recognized that particle characterization of the raw material is important but that characterization of the material *in vitro* or *in vivo* is much more useful for predicting biological effects. Bouwmeester *et al.* (2010) suggest doing a very broad characterization suite for the “as-supplied” material and then a more narrow, material and end-point-specific suite of characterization techniques for the material within the biological system.

Lack of material characterization greatly limits the number of studies that contribute to our understanding of this subject and precludes our ability to make interstudy comparison of results. Characterization methods are discussed further in Chapter 2. Despite these challenges, we can make several general conclusions about factors affecting oral bioavailability of small particles. Particle size and material are important for mechanism, site, and extent of uptake. Surface charge and hydrophobicity influence mucin interactions and paracellular transport. Surface coatings and protein interactions can alter particle behavior and site of uptake by influencing agglomeration and cell recognition. *In vivo* studies have shown us that particle size, surface charge, and dissolution kinetics will influence overall bioavailability and distribution. While this limited understanding is a far cry from our ambition to predict oral bioavailability and distribution based on a list of particle characteristics, our knowledge will continue to expand as experimental standards and minimal particle characterization requirements improve for all nanoparticle studies.

RELATED ARTICLES

Chapter 1
Chapter 2
Chapter 3

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Nanomedicine in Cancer Treatment

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1 INTRODUCTION

Cancer is a major death-causing disease worldwide and one in four deaths in the United States is reported to be due to cancer. Despite remarkable advances in our understanding of cancer and its treatment, it is estimated that, in 2013, there will be 580,350 cancer-related deaths in the United States, corresponding to almost 1600 deaths per day (Siegel, Naishadham, and Jemal, 2013). Although statistical reports indicate that cancer death rates have decreased from their peak, reflecting improvements in the early detection and/or treatment of cancer (Berry *et al.*, 2005; Edwards *et al.*, 2010; Etzioni *et al.*, 2008), high mortality projections are a clear indication that further progress for therapeutic regimens are needed. Such advances would derive from improved early screening and diagnosis, as well as targeted therapies that selectively target tumor cells and have reduced off-target toxicity; these two nanomedicine-based approaches are likely to have a considerable future impact.

Nanomedicine is a subfield of nanotechnology and is basically the medical application of nanoscale (1–100 nm) constructs built on nanotechnology standards, such as the control, repair, and construction of human biological systems (<http://www.nano.gov>). These technological innovations have the potential to turn molecular findings arising from genomics and proteomics into widespread benefits for patients. Past diagnostic

and therapeutic approaches relied primarily on invasive medical procedures (i.e., random biopsy and surgery) and nonspecific remedies such as irradiation and chemotherapy. To improve the efficiency of treatment and promote the welfare of cancer patients, attempts to apply Paul Ehrlich's "magic bullet" concept (Strebhardt and Ullrich, 2008) to chemotherapy using nanoparticles are pivotal within the field of nanomedicine. Nanoparticles can be designed to integrate a variety of chemotherapeutic agents and to target the tumor site directly and specifically for better effectiveness and lower toxicity (Brannon-Peppas and Blanchette, 2004; Egusquiaguirre *et al.*, 2012; Kim *et al.*, 2010; Koo, Rubinstein, and Onyuksel, 2005; Wang, Langer, and Farokhzad, 2012; Yu *et al.*, 2010). Furthermore, nanocarriers can assist drug solubilization and protect the drug from degradation by manipulating the size and surface properties to sidestep their opsonization, thus maintaining prolonged blood circulation (Lindner *et al.*, 2004; Moghimi, Hunter, and Murray, 2001; Senthilkumar, Mishra, and Jain, 2008; Torchilin, 1998; Yoo, Chambers, and Mitragotri, 2010). These operative properties of nanoparticles have made them one of the most actively researched platforms in nanomedicine.

In this chapter, recent concepts, principles, and studies concerning the applications of nanomedicine in cancer therapy will be summarized, focusing on the principle of cancer treatment using nanomaterials in addition to the unique and critical

properties of nanoparticles that discriminate them from other types of cancer therapeutics and make them better candidates than the current therapeutic strategies for specific types of cancer.

2 CONCEPTS AND PRINCIPLES OF CANCER TREATMENT USING NANOMATERIALS

2.1 Passive Targeting

Passive targeting of nanomaterials and accumulation of their drug cargo in tumor tissues can be attributed mainly to their prolonged circulation time and enhanced permeability and retention (EPR) effect. The EPR effect is the tendency of a certain size of molecule to accumulate in tumor tissue more than that in normal tissue (Iyer *et al.*, 2006). As tumor tissue has a diffusion limit at a volume of 2 mm³ or above, nutrition supply, waste excretion, and oxygen consumption are obstacles that must be overcome. Tumors clear these hurdles by increasing the surrounding vasculature in a process called

angiogenesis (Figure 1) (Risau, 1997). Aberrant distortion, basement membrane abnormality, and a lack of pericytes lining the endothelial cells are the characteristics of angiogenesis (Baban and Seymour, 1998; Risau, 1997). Because of this imperfect tumor vasculature, vessels are enclosed with leaky gap sizes of 100 nm to 2 μ m depending on the type of cancer (Figure 1) (Hobbs *et al.*, 1998; Shubik, 1982). In addition, as tumors lack a well-defined lymphatic system, interstitially accessed drugs and nanoparticles have higher retention times in tumor tissues than in normal tissues, because the EPR effect results from the combination of leaky vasculature and poor lymphatic drainage (Figure 1) (Haley and Frenkel, 2008). Nanoparticles smaller than the vascular fenestrations can cross the interstitium and accumulate in the tumor (Figure 2a) (Haley and Frenkel, 2008).

2.2 Active Targeting Strategies

Active targeting implies the use of conjugated targeting moieties that identify receptors or proteins

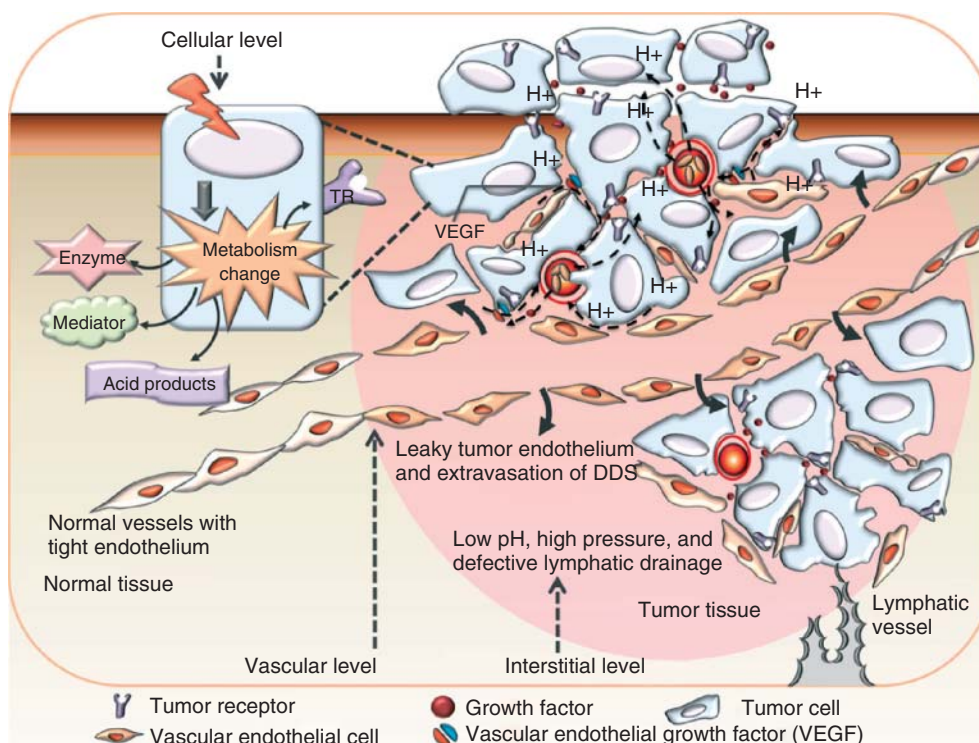


Figure 1. Cellular, vascular, and interstitial levels of tumor tissue. DDS: Drug delivery system; TR: Tumor receptor.

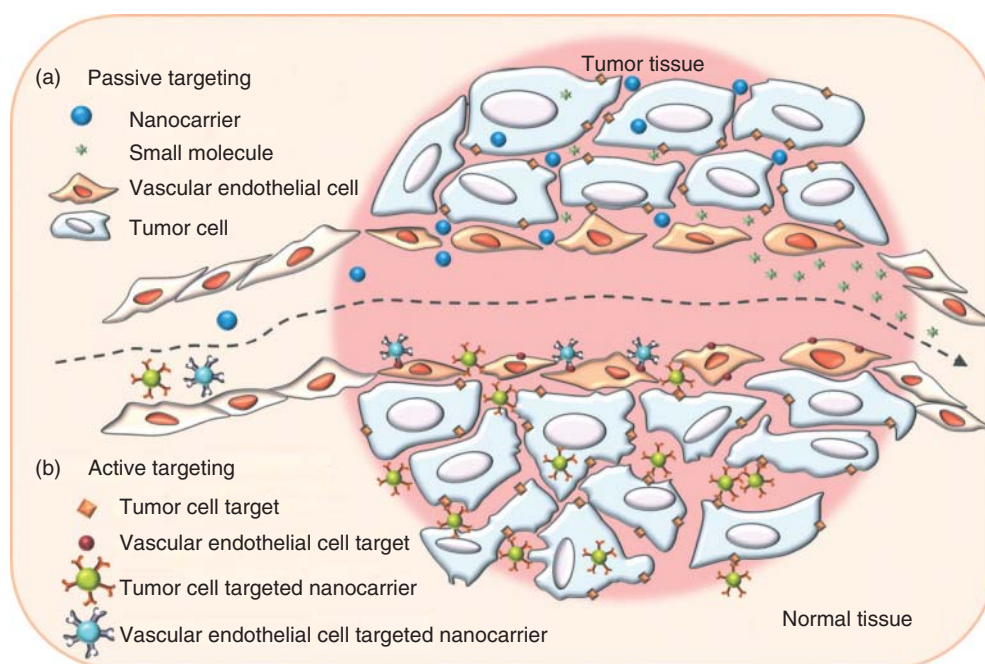


Figure 2. Concept of passive and active targeting. (a) Passive targeting of nanocarriers. Nanocarriers reach tumors selectively through the leaky vasculature surrounding the tumors. Schematic representation shows the influence of size on retention in the tumor tissue. Small molecular therapeutics (green) diffuse freely in and out the tumor blood vessels because of their small size and thus their effective concentrations in the tumor decrease rapidly. In contrast, because of large size, drug-loaded nanocarriers (blue) cannot diffuse back into the blood stream, resulting in progressive accumulation: the EPR effect. (b) Active targeting strategies. Target-ligand grafted at the surface of nanocarriers bind to receptors (over)expressed by cancer cells or vascular endothelial cells.

expressed in tumor cells, known as *tumor-associated antigens (TAAs)* (Haen and Rammensee, 2013), for enhanced and/or targeted delivery (Byrne, Betancourt, and Brannon-Peppas, 2008). This approach was first conducted using liposomes in the 1980s by covalently linking a specific antibody (referred to as an *immunoliposome*) to enhance targeted delivery (Huang, Huang, and Kennel, 1980). This strategy was readily used by researchers in the field of drug delivery using nanoparticles, and it has become one of the most well-established strategies for enhancing the efficacy of nanomedicine (Cardoso, Peca, and Roque, 2012; Li and Huang, 2008). The moieties for conjugating nanoparticles are usually ligands or targeting antibodies because of their high specificity and high affinity for either tumors or their associated endothelium, which consequently maximize the localization and accumulation in the tumor (Figure 2b). For these reasons, antibody targeting is regarded as a promising targeting strategy. However, several studies have reported that antibody targeting does not increase tumor

localization; rather, it increases internalization only (Hatakeyama *et al.*, 2007; Kirpotin *et al.*, 2006), meaning that biodistribution still relies on passive targeting to tissues with loose vasculature, and that targeting moieties only trigger endocytosis after extravasation into the tumor. Through the EPR effect, a long circulation time would allow a predominant accumulation of nanoparticles to the tumor site, followed by the enhancement of endocytosis induced by the targeting molecules. Whatever the detailed mechanisms, active targeting using targeting moieties increases the delivery of therapeutics to the specific tumor site. Although not fully elucidated, the most likely transport mechanism of nanoparticles into the cells is via endocytosis, and the nanoparticle-induced internalization of the drugs after attachment is crucial to ensure the potency of some anticancer drugs, especially in gene delivery, siRNA silencing, and other biotherapeutics (Nielsen *et al.*, 2002).

There are several targeting moieties used for active targeting. Among them, folate is the most widely

used small organic molecule. Folate has a high affinity for the folate receptor, which is significantly overexpressed in many cancer cells compared to normal tissue, in some cases by 100–300 orders of magnitude (Ross, Chaudhuri, and Ratnam, 1994). The anti-HER2-antibody trastuzumab (Herceptin), which targets the human epidermal growth factor receptor 2 (HER2) and was found to be overexpressed in one-fourth of breast cancers (Pegram, Konecny, and Slamon, 2000), also exploits a targeting moiety. Recently, liposomes encapsulating topotecan, a topoisomerase-I inhibitor, were functionalized with an anti-HER2 antibody for targeting breast tumors. HER2-targeted liposomes showed a more than twofold enhancement of antitumor activity compared with nontargeted liposomes, and a fivefold enhancement compared with free topotecan (Drummond *et al.*, 2010). The cyclic peptide cRGD (Arg-Gly-Asp-D-Phe-Lys) has also been studied as a potential candidate for active targeting of nanoparticles because of its high affinity for $\alpha_v\beta_3$, which is overexpressed in blood vessels undergoing angiogenesis (Khemtong *et al.*, 2009).

2.3 Controlled Release Strategies

In addition to targeted delivery, developing strategies of controlled release of chemotherapeutics to reduce their adverse effect on normal tissues (Table 1) is also important. When developing a nanoparticulate delivery system, it is important to consider both drug release and biodegradation. In general, the drug release rate relies on (Singh and Lillard, 2009):

1. Solubility of the drug formulation;
2. Dissociation of the surface-bound or adsorbed drug from the carrier;
3. Drug diffusion from the nanoparticle matrix;
4. Degradation of the nanoparticulate carrier.

In other words, the drug release process is governed by the solubility, diffusion, and biodegradation of the particle matrix. As previously mentioned, the first factor to consider is that the method of incorporation could affect the drug release profile. In the case of a drug homogeneously distributed throughout the nanoparticle, if the drug diffusion is dominant compared to the matrix erosion, the diffusion rate acts as a determining factor. Weak binding or adsorption of drugs to the relatively large surface of nanoparticles leads to a rapid, initial release, or burst effect (Magenheim, Levy, and Benita, 1993). For example, drugs incorporated into colloidal carrier formulations show sustained release characteristics instead of rapid release (Fresta *et al.*, 1995). In the case of drugs coated with polymers, the diffusion rate across the polymer becomes a determining factor. In addition to this, ionic interactions between the drug and the ancillary ingredients affect the drug release, because the two components can form complexes. For example, Fe(III), which retards the release of doxorubicin (DOX), was co-incorporated into nontoxic and biodegradable albumin-dextran sulfate microspheres to reduce their cardiotoxicity (Chen, McCulloch, and Gray, 1994). On the other hand, the competitive electrostatic interactions of the ethylene oxide–propylene oxide block copolymer (PEO-PPO) with chitosan are exploited for rapid drug release (Calvo *et al.*, 1997).

Likewise, nanocarriers must release their entrapped cargo at a rate that is appropriate for reaching the therapeutic concentrations of the drug and maintaining the therapeutic range of free-form drug concentrations. One current research aim is to tailor the release of drugs from nanoparticles with the hope of rapidly exposing the tumor after extravasation. In this context, several drug release strategies have been adapted successfully:

Table 1. Major adverse effects of conventional chemotherapeutics.

Chemotherapeutic	Systemic adverse effect	References
Paclitaxel (PTX)	Allergy (by Cremophor EL used for vehicle)	(Gelderblom <i>et al.</i> , 2001)
Cisplatin	Nephrotoxicity	(Uchino <i>et al.</i> , 2005)
Doxorubicin (DOX)	Cardiotoxicity (Cardiomyopathy)	(Zhang <i>et al.</i> , 2009)
Fluorouracil (5-FU)	Myelosuppression, mucositis, and neurotoxicity	(Pirzada, Ali, and Dafer, 2000)
Camptothecin (CPT)	Myelosuppression	(Sonis <i>et al.</i> , 1997)
Cytarabine	Cerebellar toxicity (Ataxia), leukopenia, anemia, and thrombocytopenia	(Pizzolato and Saltz, 2003)
		(Schiff <i>et al.</i> , 2009)

1. pH triggered release;
2. Temperature triggered release;
3. Enzyme-sensitive release from carrier.

Tumors tend to have lower pH values than physiological levels because of their heavy dependence on glycolysis (Figure 1). Talelli and colleagues developed a pH-sensitive polymer micelle with a covalently entrapped DOX that could release a 200-fold amount of drugs at the tumor site (Talelli *et al.*, 2010). Therapy using drugs encapsulated in thermosensitive liposomes in combination with hyperthermia therapy (Merlin, 1991) showed an enhancement of therapeutic efficacy for many antitumor drugs such as DOX, cisplatin, and methotrexate (Weinstein *et al.*, 1980). Experiments involving enzyme-triggered release using tumor-associated secretory phospholipase A2s (PLA₂) were conducted in an MT-3 xenograft model with sPLA₂ degradable liposomes encapsulating cisplatin. A significant reduction of the tumor growth was observed, suggesting an increase in cisplatin bioavailability because of sPLA₂-mediated degradation of the liposome carrier (Andresen, Jensen, and Jorgensen, 2005).

3 NANOMATERIALS USED FOR CANCER THERAPY

Nanoparticulate drug carriers are solid molecular assemblies with sizes in the nanometer range, and may or may not be biodegradable. The most commonly used nanocarriers in current preclinical and clinical tumor treatments are illustrated in Figure 3; they have capsule (e.g., polymeric, metallic, or ceramic nanoparticles), hyperbranched molecule (i.e., dendrimers), or amphiphilic core/shell (e.g., polymeric micelles and liposomes) structures (Danhier, Feron, and Preat, 2010). Nanocarriers physically entrap the drug in their loading space, protecting it from damage in the biological environment from processes such as enzymatic inactivation, degradation, and phagocytosis. When deciding on or designing a nanocarrier, a variety of factors should be considered (Shapira *et al.*, 2011):

- Degree of complexity of its synthesis or preparation;
- Cost of nanocarrier formation;
- Quality or quantity of loading capacity of different therapeutics;
- Stability of the loaded nanoparticle in solution before use;

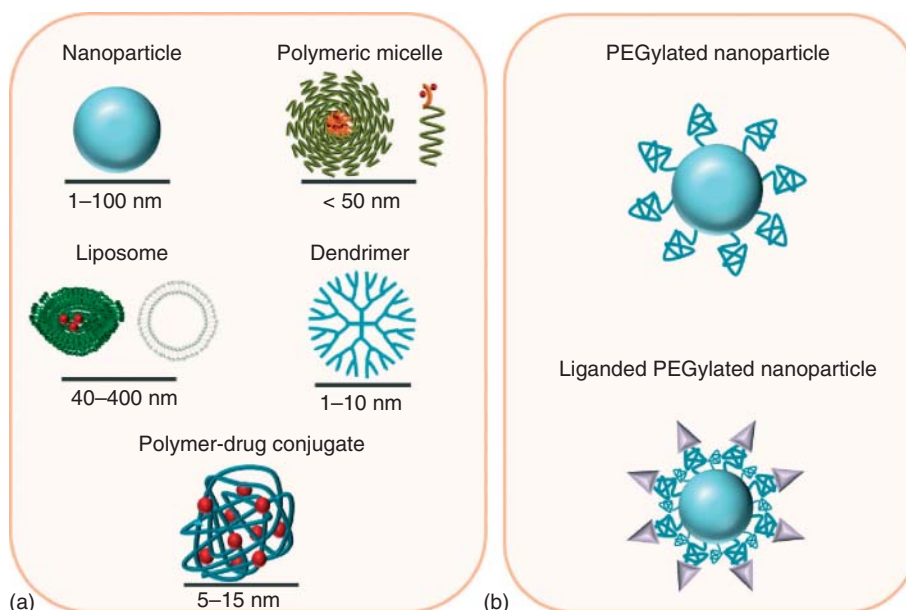


Figure 3. Nanoparticles used in drug delivery. (a) Types of nanocarriers. (b) Schematic representation of PEGylation and ligand grafting.

- Drug administration route;
- Half-life in the circulation;
- Bioaccessibility to the target tissue;
- Selectivity of its biodistribution;
- Biological fate (e.g., degradability).

As four of these factors must be determined by precise pharmacokinetic and pharmacodynamic studies, there are only a few nanocarriers that have been approved by the US Food and Drug Administration (FDA) and European Medicines Agency for cancer therapy (Bharali and Mousa, 2010). However, because of the potential to improve the shortcomings of the existing treatment methods, many nanocarriers are currently being studied or are in clinical trial/preclinical development (Zhang *et al.*, 2008b).

3.1 Nanoparticles

Nanoparticles (Figure 3a) are solid, spherical structures, ranging from approximately 1 to 100 nm in size; drugs are encapsulated within or coated on their surface. Metallic (e.g., magnetic nanoparticle and gold nanoparticle (GNP)) and ceramic (silica) nanoparticles are usually used for drug delivery or multifunctional theragnostics. They can be PEGylated and grafted with targeting ligands (Figure 3b).

3.1.1 Magnetic Nanoparticles

Magnetic nanoparticles are garnering great interest because of their specific properties, and are applied in magnetic field targeting (Chertok, David, and Yang, 2010), MRI imaging (Liu *et al.*, 2010), and hyperthermia therapy (Ma *et al.*, 2012). The use of magnetic nanoparticles as drug delivery carriers was first reported by Widder and colleagues in 1983 in an attempt to deliver DOX to sarcoma tumors implanted in rat tails (Widder *et al.*, 1983). Subsequently, many studies using magnetic nanoparticles for cancer targeting have been conducted. Furthermore, because of the development of synthetic methods for obtaining highly crystalline and monodisperse magnetic nanoparticles (Hyeon *et al.*, 2001), the number of studies of potential applications of magnetic nanoparticles in the medical field has increased. Carboplatin-loaded chitosan magnetic nanoparticles

were found to effectively generate heat in tumor interstitium by static magnetic targeting, and to facilitate apoptosis (Li *et al.*, 2009). Ferrofluid (FF) nanoparticles consisting of colloidal dispersions of iron oxides and hydroxides using a starch polymer could be concentrated on tumor region by external magnetic field (Alexiou *et al.*, 2003). Superparamagnetic CaCO_3 mesocrystals, in which mesocrystalline CaCO_3 particles encapsulated DOX, Au-DNA, and Fe_3O_4 silicananoparticles, were designed and synthesized. In this study, the delivery using this multistage superparamagnetic CaCO_3 mesocrystal was tenfold more efficient than that of Au-particle themselves (Zhao *et al.*, 2010).

Although the versatile applicable field of magnetic nanoparticles in nanomedicine is powerful, potential issues related to their use in theragnostics have been suggested (Pankhurst *et al.*, 2003):

- Embolism due to magnetic carrier accumulation;
- Difficulty in scaling up from animal models because of the large distance between the magnet and the target site;
- Undesirable toxicities due to the accumulated magnetic carrier;
- Interrupted attraction of the drug to the magnetic field after release.

3.1.2 Gold Nanoparticles

GNPs have recently emerged as attractive drug carriers for delivery into tumors (Paciotti *et al.*, 2004) (See also Chapter 8 in this book). They have unique physical and chemical properties. First, the gold itself is essentially inert and nontoxic (Connor *et al.*, 2005). Second, GNPs are easily synthesized. For example, size-dependent monodisperse nanoparticle synthesis can be achieved according to published synthetic methods (Carotenuto and Nicolais, 2003; Connor *et al.*, 2005). Third, GNPs can be easily functionalized through thiol linkages, and several research groups have fabricated GNP delivery systems bearing functional moieties. Furthermore, their photophysical properties make the manipulation of drug release possible, even at distant sites (Skirtach *et al.*, 2006). In photo-induced thermal therapy, GNPs are directed by specific ligands or antibodies to the cancer cells to kill them selectively. Several hyperthermia therapies have been developed by loading GNPs into functional nanocarriers such as

chitosan-conjugated or pluronic-based nanocarriers. It has been reported that an intravenous (iv) injection of GNP-loaded nanocarriers induced efficient thermolysis *in vivo* following complete tumor removal without damage to the surrounding tissue (Choi *et al.*, 2011). A comparison study using PEGylated GNPs demonstrated that short polyethylene glycol (PEG) chains in combination with a small core size displayed the best targeting and permeation efficiencies to the brain microvasculature (Etame *et al.*, 2011). Targeting moieties, including anti-HER2 antibodies (Hainfeld *et al.*, 2011), RGD-peptide (Xie *et al.*, 2011), transferrin (Tf) (Choi *et al.*, 2010), and tamoxifen (estrogen receptor antagonist), linked to PEGylated GNPs showed more successful cancer therapeutic results than other systems (Dreaden *et al.*, 2009). In addition to targeted therapy, thermal ablation therapy in combination with imaging using near infrared (NIR) absorbable core/shell (silica/gold) nanoparticles is an active research field (Gobin *et al.*, 2010). Several GNPs are in clinical trial because of their multiple attractive features such as triple modality theragnostics (i.e., drug delivery, imaging, and thermo generation), convenient functionalization with thiol groups, and biocompatibility; thus, the application of GNPs in cancer treatment should increase (Duncan, Kim, Rotello, 2010; Kennedy *et al.*, 2011).

3.2 Polymeric Micelles

Polymeric micelles (Figure 3a) are assembled in spheroidal structures with a hydrophobic core and a hydrophilic corona. The core acts as a reservoir for hydrophobic drugs, improving their poor solubility, whereas the corona allows a long circulation time of the encapsulated drug by separating the core from the blood components (Kedar *et al.*, 2010). Polymeric micelles are dynamic systems formed by self-assembly as a result of hydrophobic or ionic interactions between polymers, and have a variable size, usually below 50 nm (Park *et al.*, 2008). The building blocks of polymeric micelles are usually block copolymers or graft polymers. Block copolymers are formed with blocks of differently polymerized monomers that have different physicochemical properties, and among them, the AB type is the most favorable for the synthesis of a well-defined size, shape, and diameter distribution.

Graft copolymers are branched polymers in which the side chains are structurally distinct from the main chain (Jeong *et al.*, 1997).

There are two methods for encapsulating drugs into the inner core of the polymeric micelles, namely chemical conjugation and physical entrapment. Chemical conjugation uses covalent bonds, such as amide bonds, between the drug and the hydrophobic polymer core. Because of steric hindrance, this bond is resistant to enzymatic cleavage (Ulbrich *et al.*, 1987). Physical entrapment methods include dialysis (Figure 4a) and an oil-in-water emulsion process (Figure 4b). On the basis of a comparison study using DOX, the dialysis method has the advantage of not using toxic solvents, whereas the oil-in-water method has the advantage of more efficient drug incorporation than the dialysis method (Kwon *et al.*, 1995, 1997).

Recently, in clinical application, polymeric micelles have attracted great scientific interest (Kedar *et al.*, 2010), not only for their targeting properties, but also for their additional advantages including solubility and the capability of P-glycoprotein inhibition (Gong *et al.*, 2012). Because of these advantages, many clinical trials use polymeric micelle drug carrier systems. For example, Genexol-PM, paclitaxel (PTX)-incorporated micelles using the poly(ethylene glycol)-*b*-poly(DL-lactide) block copolymer, improved the side effects of a conventional PTX formulation (Taxol) in terms of toxicity. Originally, because of the large amount of the surfactant Cremophor EL it contained, taxol caused substantial side effects resulting in toxicity (Table 1). In the case of Genexol-PM, the high solubility of micelles was used to mitigate the side effects of the conventional PTX formulation (Lee *et al.*, 2008; Park *et al.*, 2004). From the targeting point of view, Matsumura and colleagues reported that NK-105, which consists of PTX incorporated in a PEGylated micelle, showed high tumor-selective delivery with low toxicity in phase I clinical trials (Hamaguchi *et al.*, 2007; Matsumura and Kataoka, 2009). Kabanov *et al.* (2002) reported a multidrug resistance (MDR) circumvention study using a Pluronic formulation of DOX, known as *SP-1049C*, and emphasized the unique advantage of Pluronic micelles that was not displayed by any other synthetic polymer (Danson *et al.*, 2004; Kabanov, Batrakova, and Alakhov, 2002).

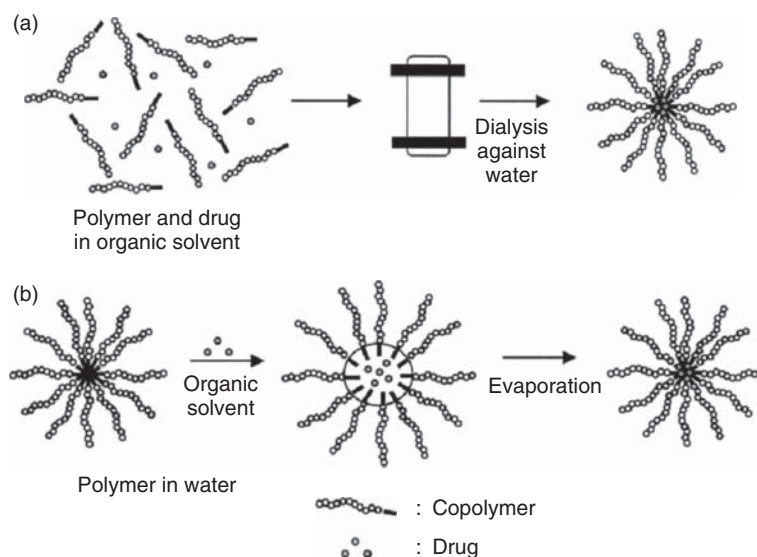


Figure 4. Drug loading of polymeric micelles. (a). Dialysis method. (b). Oil-in-water methods. [Jones and Leroux, 1999; La *et al.*, 1996. Reproduced by permission of Elsevier.]

Although polymeric micelle systems are hampered by the issues of micellar instability and premature drug release (Kim and Park, 2010), several modifications have been developed to overcome those drawbacks, including chemical modifications of the micellar core such as cross-linking (Gao and Uludag, 2001; Kakizawa, Harada, and Kataoka, 2001), partial crystallization through stereo-complex formation (Kang *et al.*, 2005), and the introduction of drug compatible moieties (Lavasanifar, Samuel, and Kwon, 2002; Nakanishi *et al.*, 2001; Yokoyama *et al.*, 1998). Advantages and disadvantages of polymeric micelles are listed in Table 2.

3.3 Liposomes

Liposomes (Figure 3a) are closed spherical vesicles formed by lipid bilayers encapsulating an aqueous core. Hydrophilic drugs that cannot readily pass through the lipid membrane can be entrapped in this aqueous core. Furthermore, hydrophobic chemicals can be incorporated into the membrane, enabling the liposome to carry both hydrophobic and hydrophilic drugs. The lipid bilayer of the liposome can fuse with cell membranes, thus delivering the liposome contents. Liposomes usually reach the tumor site by extravasation through passive targeting or active targeting because they can easily be manipulated

Table 2. Advantages and disadvantages of polymeric micelles as a drug delivery system.

Advantages	Disadvantages
• Small diameter with narrow distribution (10–100 nm) • High water solubility • Low toxicity • Separated functionality (multifunctional design possible) • P-glycoprotein inhibitory activity (Dintaman and Silverman, 1999) • Improved controlled release functions • Suitable for intravenous administration	• Difficulty of block copolymer synthesis on a large industrial scale in a highly reproducible manner • No universal incorporation method • Lack of stability in blood • Limited number of polymers for use • Lack of suitable methods for scale-up

Modified from (Yokoyama, 2010; Lu and Park, 2013).

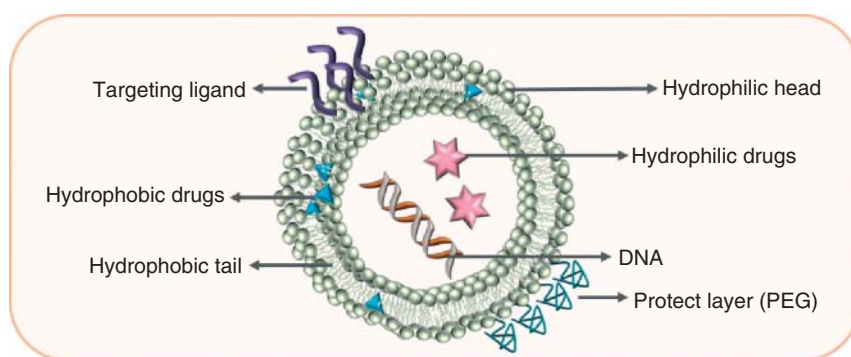


Figure 5. Diagram of a liposome. The hydrophobic region captures the drugs in the central core when the liposomes are synthesized. The outer surface can be functionalized with ligands for active targeting or protected by PEGylation.

through targeting ligands (Figure 5). They can also be PEGylated to avoid clearance by the mononuclear phagocytic system (MPS). As liposomes range from 40 nm to about 400 nm in size, they can be rapidly cleared by the MPS, which phagocytoses particles larger than 30 nm. Opsonization by the innate immune system, which marks particles for ingestion and destruction by the MPS (Owens III and Peppas, 2006), is reduced by PEGylation and, consequently, PEGylation increases the circulation half-life of liposomes (Malam, Loizidou, and Seifalian, 2009).

During recent years, liposomes have been studied with growing success as pharmaceutical carriers, and some liposomal formulations of cancer therapeutics have already been approved for human use. The therapeutic index of DOX was improved by incorporation into liposomes, resulting in less cardiotoxicity and equal anticancer activity. Doxil® (Alza Pharmaceuticals, San Bruno, CA, USA), also known as *Caelyx*® outside the United States, is a PEGylated liposomal DOX hydrochloride used to treat cancer in AIDS-related Kaposi sarcoma, advanced breast cancer, ovarian cancer, and multiple myeloma (Barenholz, 2012; O'Brien *et al.*, 2004). However, this PEGylated liposomal DOX has a side effect called *palmar plantar erythrodysesthesia* (PPE), known as *hand-foot syndrome* (Gordon *et al.*, 1995). Small amounts of Doxil® can ooze from capillaries in the palms of the hands and soles of the feet, resulting in redness, tenderness, tingling, and peeling of the skin, which can be painful. Patients (50.6%) treated with Doxil® at a dose of 50 mg m⁻² every 4 weeks developed hand-foot syndrome in clinical testing (Vergote *et al.*, 2009). Myocet™

(Elan Pharmaceuticals, Inc., Cedar Knolls, NJ, USA), which is used to treat metastatic breast cancer in combination with cyclophosphamide, is a non-PEGylated liposomal DOX, and therefore does not cause hand-foot syndrome. In a randomized cardiotoxicity comparison trial of Myocet™ with conventional DOX, 2% of the Myocet™-treated group developed congestive heart failure, whereas 8% of the conventional DOX-treated group developed this condition (Harris *et al.*, 2002). However, Myocet™ provides a limited degree of prolonged circulation compared with the free drug because of the non-PEGylation, although the liposome encapsulation significantly alters the biodistribution of DOX, resulting in some reduction of the toxicity associated with a decreased frequency of cardiotoxicity and neutropenia (Batist *et al.*, 2001). Liposomal daunorubicin DaunoXome® (Gilead Sciences, Inc., Forest City, CA, USA), which has a very small size (~45 nm) and a rigid bilayer, provides extended circulation and is effective against Kaposi's sarcoma and other types of tumors including neuroblastoma and acute myeloid leukemia (AML) (Fassas and Anagnostopoulos, 2005; Styczynski, 2013). DaunoXome® shows a markedly prolonged circulation and enhanced tumor accumulation capability because of a series of modifications to the liposome structure that impede uptake by MPS (Gill *et al.*, 1996).

3.4 Dendrimers

Dendrimers are repeatedly branched polymeric macromolecules with the shape of many branched

arms extending from a center, resulting in a controlled three-dimensional architecture (Figure 3a). They are nanometer-sized (1–10 nm) and consist of a central core, branching units, and terminal functional groups. They are produced by two major types of synthetic methods: divergent methods (Tomalia-type) and convergent methods (Fréchet-type), which differ in the direction of synthesis. Divergent methods synthesize outward from the core, whereas convergent methods synthesize inward toward the core (Tomalia and Fréchet, 2002). Convergent methods have the advantage over divergent methods in preventing the generation of impurities, that is, branches that are shorter than the others. However, divergent methods are more widely used than convergent methods because overcrowding due to steric effects along the core is a limiting factor (Tomalia and Fréchet, 2002). Polyamidoamine (PAMAM) dendrimers are synthesized by divergent methods, whereas polypropylenimine (PPI) and polyaryl ether dendrimers are synthesized by convergent methods (Bharali *et al.*, 2009). Recently, efforts have focused on the synthesis and preclinical evaluation of a multipurpose PAMAM dendrimer that can be used for diagnostic imaging (Wiener *et al.*, 1994) and drug delivery. Dendrimers have the advantages as nanodevices for cancer therapy, such as (Bharali *et al.*, 2009; Tomalia, Reyna, and Svenson, 2007):

- Feasible topology: feasibility of a precise control over size or shape very close to important cellular components, such as proteins and DNA;
- Functionality: numerous terminal surface groups suitable for conjugation of drugs, targeting moieties, and biocompatible groups;
- Dimensions: interior empty space for encapsulating therapeutics and imaging molecules, enabling the reduction of toxicity and controlled drug release.

In the last few years, the multifunctional nature of dendrimers has facilitated the simultaneous incorporation of both therapeutics and targeting moieties. A folate receptor-targeted dendrimer showed increased targeting efficiency and was less toxic than the free drug at equal cumulative doses (Kukowska-Latallo *et al.*, 2005). Fluorouracil (5-FU)-conjugated DNA assembled in a PAMAM dendrimer targeting the folate receptor also minimized the toxicity of 5-FU (Choi *et al.*, 2005). Likewise, certain peptides (Li

et al., 2010), polymers (Okuda *et al.*, 2006), sugar derivatives (Medina *et al.*, 2011), aptamers (Lee *et al.*, 2011), and antibodies (Shukla *et al.*, 2006) can be complexed with dendrimers to enhance their retention and accumulation in tumor sites. However, despite these numerous benefits, dendrimers show generation- and concentration-dependent hemolytic and hematologic toxicities (Jain *et al.*, 2010). These toxicities are attributed to the interaction between the surface cationic charge of the dendrimers and the negatively charged biological membranes *in vivo*. Furthermore, the accumulation of dendrimers in the liver and the kidney also slowed the clinical utilization of these carriers (Duncan and Izzo, 2005). Two strategies have been utilized to overcome those toxicities: first, the design and synthesis of biocompatible and degradable dendrimers; and second, masking of the surface charge of the dendrimers by tailoring of the dendrimers themselves. The evolution of novel dendrimers with biocompatible components, and the surface modification of commercially available dendrimers by PEGylation (Chen *et al.*, 2004), acetylation (Kolhatkar *et al.*, 2007; Zhuo, Du, and Lu, 1999), glycosylation (Kim *et al.*, 2007), and amino acid functionalization have been proposed as useful strategies to solve the nanosafety problems of dendrimer-based nanotherapeutics (Cheng *et al.*, 2011).

3.5 Polymers

Polymers are one of the most commonly used materials as drug delivery carriers (Langer and Peppas, 2003). Polymer–drug conjugates (Figure 3a) are polymeric macromolecules constituted by a polymer backbone on which drugs are conjugated via linker regions. They can also be grafted using targeting ligands (Duncan, 2006). In addition to this, generally, cationic polymers are widely accepted as gene therapy carriers because of their ability to interact with cells and to achieve efficient condensation with DNA in virtue of the charge interaction between positively charged polymers and negatively charged cellular membrane and DNA phosphate backbone. Polymer/DNA complexes are called *nanospheres* because they are matrix systems in which the drug is dispersed throughout the particles physically and uniformly, whereas liposomes are called *nanocapsules* because they are vesicular systems in which the drug is entrapped in a cavity

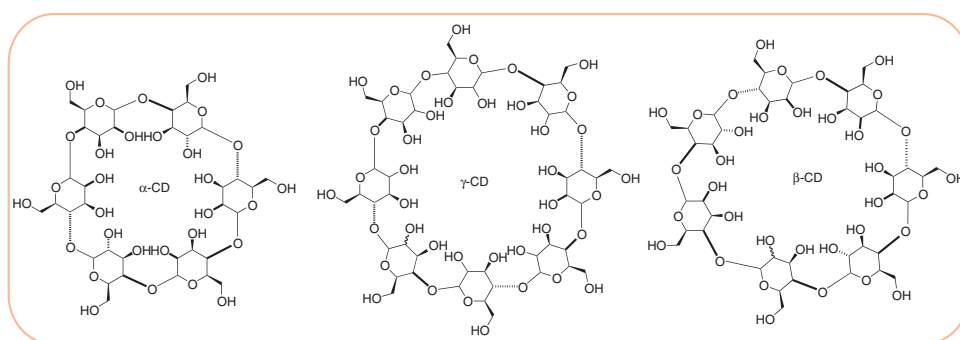


Figure 6. Chemical structures of CDs. CDs composed of 6, 7, and 8 glucose units are called α -, β -, and γ -CDs, respectively.

surrounded by a polymer membrane (Hillaireau and Couvreur, 2009). Polymer/DNA complexes are more stable than those involving cationic lipids and protect the DNA against nuclease degradation (Jiang *et al.* 2007a, b). In this chapter, polymers such as cyclodextrin (CD), chitosan, polyethylenimine (PEI), poly(β -amino ester)s (PAEs), and their derivatives are summarized.

CDs, which have been approved by the FDA as food additives, are naturally occurring cyclic oligosaccharides derived from the enzymatic degradation of starch (Ortiz Mellet, García Fernández, and Benito, 2011). Compositions of CDs with 6, 7, and 8 glucose units are called α -, β -, and γ -CDs, respectively. Chemical structures of α -, β -, and γ -CDs are shown in Figure 6. CDs improve the cellular uptake of genes and also delay their degradation by increasing their stability against endonucleases, because of their favorable physicochemical and biological properties and unique capability of forming inclusion complexes with small, hydrophobic molecules in their inner basket-shaped cavities (Forrest, Gabrielson, and Pack, 2005). Natural CDs and their derivatives have been used in drug delivery (Loftsson *et al.*, 2005; Uekama, Hirayama, and Irie, 1998) as well as gene delivery systems (Challa *et al.*, 2005; Dass, 2002; Redenti *et al.*, 2001).

Chitosan is a linear cationic polysaccharide derived from chitin, a polymer abundant in nature. It is reported to be a biocompatible, biodegradable polycationic polymer with low immunogenicity and low cytotoxicity compared to PEI (Mansouri *et al.*, 2004). Therefore, chitosan and its derivatives were applied as potentially safe cationic carriers for aerosol gene therapy in K-ras^{LA1} lung cancer model mice. They delivered programmed cell death 4 (*PDCD4*) (Jin *et al.*, 2006) or phosphatase and tensin (*PTEN*) homolog (Jin *et al.*, 2008) as therapeutic suicide genes for lung cancer therapy and

showed suppression of lung cancer and induction of apoptosis.

In 1995, for the first time, Behr's group used PEI for the delivery of oligonucleotides. Since then, PEI has attracted much attention owing to its high transfection efficiency (Boussif *et al.*, 1995). The reason for the high transfection ability of PEI is hypothesized to be its high buffering capacity, which is called the *proton sponge effect*, although this matter is highly debated (Akinc *et al.*, 2005; Benjaminsen *et al.*, 2013). In addition, PEI displays high density of positive charges in virtue of its high content of primary amino groups, enabling the chemical coupling of targeting moieties or intracellular active components and a tight compaction of nucleic acids. PEI exists in two forms with a wide range of molecular weights: a branched form and a linear form as shown in Figure 7 (Lungwitz *et al.*, 2005). However, because of its high molecular weight and high density of positive charge, PEI displays high cytotoxicity. In cancer gene therapy, to reduce PEI cytotoxicity, the surface charge of PEI has been masked by conjugating hydrophilic molecules such as PEG (Petersen *et al.*, 2002) or its reduced molecular weight (Godbey, Wu, and Mikos, 2001; Kunath *et al.*, 2003).

PAEs are also one of the widely studied biodegradable cationic gene carriers because of their low cytotoxicity and potential application in the timely release of DNA inside transfected cell (Lim *et al.*, 2000, 2002; Luo and Saltzman, 2000). Arote *et al.* reported that folate-PEG-PAE/TAM67 (dominant-negative mutant of c-Jun) complex showed marked anti-tumor activity against folate receptor-positive human KB cell-xenografted nude mice (Arote *et al.*, 2010).

Recently, polymers are being intensively studied as gene carriers for cancer therapy because of their many desirable properties such as DNA condensation, which facilitates the cellular uptake of DNA,

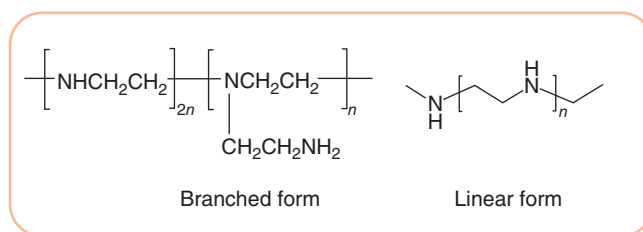


Figure 7. Chemical structures of branched and linear PEIs.

Table 3. Examples of targets for active targeting in cancer therapy.

Types of cancer	Ligand	Receptor/target	References
Small cell lung cancer	Aptamer	IGF II	(Chen <i>et al.</i> , 2008b)
Lung cancer	EGF ligand	EGFR	(Tseng <i>et al.</i> , 2007)
Prostate cancer	Anisamide	Sigma transmembrane receptor	(Li and Huang, 2006b)
	Anisamide	Sigma transmembrane receptor	(Guo <i>et al.</i> , 2012)
	Aptamer	PSMA	(Farokhzad <i>et al.</i> , 2004)
	Folate	Folate receptor	(Hattoni and Maitani, 2004)
B-lymphoma	mBAFF	BAFF receptor	(Zhang <i>et al.</i> , 2008a)
Malignant B lymphoma	Anti-CD74 antibody, LL1	CD74 receptor	(Lundberg <i>et al.</i> , 2004)
Breast cancer	Anti-HER2 antibody fragment	HER2	(Chen <i>et al.</i> , 2008a)
Liver cancer	LHRH	LHRH receptor	(Leuschner <i>et al.</i> , 2006)
	Galactose	ASGP	(Wu <i>et al.</i> , 2009; Kim <i>et al.</i> , 2012)
Colorectal cancer	ST	Guanlylyl cyclase C receptor	(Waldman <i>et al.</i> , 2006)
Brain glial cell	Anti-CD71 antibody	Transferrin receptor (CD71)	(Jelinkova <i>et al.</i> , 2003)
	Anti- α_2 -GP Ab	α_2 -Glycoprotein	(Kabanov <i>et al.</i> , 1992)
Brain tumor	F3 peptide	Nucleolin	(Reddy <i>et al.</i> , 2006)

IGF II, insulin-like growth factor 2; PSMA, prostate-specific membrane antigen (glutamate carboxypeptidase II); mBAFF, mutant B cell-activating factor belonging to the TNF family; ASGP, asialo glycoprotein; LHRH, luteinizing hormone-releasing hormone; ST, bacterial heat stable enterotoxin; α_2 -GP, α_2 -glycoprotein; F3 peptide, a peptide that specifically binds to nucleolin, which is highly expressed on the surface of both glioma cells and endothelial cells of glioma angiogenic blood vessels.

and protection of DNA from endogenous nucleases as well as efficient DNA delivery. The extensive *in vitro* and *in vivo* evaluations and characterizations of the various polymers will provide insights for a safe and efficient future clinical cancer gene therapy.

Blanchette, 2004). Much of the research regarding actively targeting nanoparticle systems have been directly associated with a specific type of cancer rather than the more ubiquitous cancer targets (Table 3).

4 CURRENT CANCER-SPECIFIC THERAPEUTIC STRATEGIES IN NANOMEDICINE

According to the report of the American Cancer Society's cancer statistics in 2013, cancers of the prostate (28%), lung and bronchus (14%), and colorectum (9%) will account for about 50% of all the newly diagnosed cancers in men, whereas breast (29%), lung and bronchus (14%), and colorectum (9%) cancers are expected to account for 51% of the estimated cancer cases in women (Siegel, Naishadham, and Jemal, 2013). In this section, current therapeutic achievements for those four types of high-incidence cancer will be discussed. Tumor targeting involves many of the targets related to uncontrolled cell proliferation and angiogenesis, and relies on the capability of the targeting agent or ligand to bind to the tumor cell surface in an appropriate manner to trigger receptor endocytosis; however, there are also other molecules that are specific to the type of cancer (Brannon-Peppas and

4.1 Breast Cancer

One of the most commonly used targeting ligands for breast cancer is the HER-2, also known as *Neu*, *ErbB-2*, *CD340*, or *p185*. HER2 is a surface protein overexpressed in approximately 30% of breast cancers (Engel and Kaklamani, 2007). Trastuzumab (marketed as Herceptin), a humanized monoclonal antibody against HER-2, was approved by the FDA in 1998 for the treatment of HER-2-positive metastatic breast cancer. Using a phage libraries technique (Becerril, Poul, and Marks, 1999), antibodies (F5 and C1) to the breast tumor cell line SK-BR-3 that bind to HER2 were identified, and research using Doxil conjugated to the F5 antibody has subsequently been conducted. An *in vivo* comparison study in mice treated with Doxil or F5-coupled Doxil showed a faster and greater regression in tumor volume for F5-containing Doxil than for unmodified Doxil (Nielsen *et al.*, 2002). Nanoparticle systems also showed promising results for targeting with HER2. *In vivo* i.v. administration

of toxin-loaded targeted poly(lactic-co-glycolic acid) (PLGA) nanoparticles to mice inoculated with HER-positive BT-474 cells resulted in significantly decreased tumor growth and systemic toxicity (Chen *et al.*, 2008a). Other nanoparticle systems using cross-linked human serum albumin nanoparticles and quantum-dot-loaded chitosan nanoparticles with absorbed siRNA also showed promising results in HER2 targeted therapy (Steinhauser *et al.*, 2006; Tan, Jiang, and Zhang, 2007).

In addition to HER2, luteinizing hormone-releasing hormone (LHRH) receptor is another target that has been widely investigated for the targeted delivery of therapeutics and imaging agents to treat localized and metastasized breast cancer. Leuschner *et al.* (2006) reported an *in vitro* and *in vivo* study using LHRH-conjugated superparamagnetic iron oxide nanoparticles (SPIONs). LHRH-conjugated SPIONs showed selective accumulation (almost ninefold) in MDA-MB-435S.luc breast cancer cells *in vitro* and accumulated in primary or metastatic breast tumor sites, whereas nontargeted SPIONs were sequestered at the reticuloendothelial system (RES) *in vivo* (Leuschner *et al.*, 2006).

4.2 Prostate Cancer

To treat prostate cancer, several target proteins were investigated, including prostate-specific membrane antigen (PSMA), sigma receptor, and folate receptor. PSMA, a class II transmembrane glycoprotein, is one of the most studied targets for prostate cancer treatment (Reiter *et al.*, 1998). PSMA-specific aptamer-conjugated docetaxel-encapsulated PEGylated PLGA inhibited tumor growth compared to nontargeted nanoparticles and free drug in xenograft models of PSMA-positive LNCaP prostate cancer cells (Cheng *et al.*, 2007; Farokhzad *et al.*, 2006). Another approach that has been used for targeting PSMA in prostate cancer uses folate ligand-modified drug carriers, because PSMA is known to bind folate (Pinto *et al.*, 1996). Folate-conjugated PEGylated distearoylphosphatidylethanolamine (f-PEG-DSPE) was used as carrier in suicide gene therapy. Intratumoral delivery of genes encoding herpes simplex virus thymidine kinase (HSV-tk) and connexin 43 (Cx 43) resulted in improved tumor growth retardation after the injection of ganciclovir (GCV) in LNCaP mice xenografts (Hattori and Maitani, 2005).

The sigma receptor, overexpressed in many malignancies including prostate cancer cells, has also been used for targeting of therapeutic nanoparticles. The sigma receptor binds haloperidol and various other neuroleptics showing a high affinity for the anisamide (i.e., analog of benzamide) moiety (Banerjee *et al.*, 2004). In a study using the DU-145 human prostate carcinoma cell line, anisamide-conjugated liposomal DOX displayed significantly higher toxicity to DU-145 than nontargeted liposomal DOX, with IC₅₀ values of 1.8 and 14 μ M, respectively. In DU-145 xenograft model mice, weekly injection of the sigma receptor-targeted liposomal DOX for 4 weeks at a dose of 7.5 mg kg⁻¹ led to a significant growth inhibition with minimal toxicity, whereas free DOX showed significant toxicity (Banerjee *et al.*, 2004).

4.3 Lung Cancer

Lung cancer is a leading cause of cancer mortality with an average 5-year survival rate of approximately 15%, and is expected to account for 26% of all female cancer deaths and 28% of all male cancer deaths in the United States in 2013 (Siegel, Naishadham, and Jemal, 2013). Commonly used lung cancer treatments have been dependent on surgery, chemotherapy, and radiation, but the approval of biological therapeutics and the development of nanomedicine have opened a new era of alternative treatments. Recently, several target molecules such as aptamers, epidermal growth factor (EGF) ligands, and anisamide have been developed (Table 3). For example, Avastin® (Genentech, South San Francisco, CA), a recombinant humanized anti-vascular endothelial growth factor (anti-VEGF) monoclonal antibody, and Iressa (AstraZeneca, London, the United Kingdom), an epidermal growth factor receptor (EGFR) inhibitor (Thatcher *et al.*, 2005), have been approved by the FDA for the treatment of non-small cell lung cancer (SCLC), metastatic breast cancer, and colorectal cancer (Ferrara, Hillan, and Novotny, 2005). However, the lack of sensitive ligands specific to lung cancer cells makes the targeting of systemically administered drug delivery systems difficult. To solve this problem, cell-based systemic evolution of ligands by exponential enrichment (cell-SELEX) has been applied to find aptamers specific for SCLC. *In vitro* studies demonstrated selective interaction

and binding of these aptamers to SCLC cells but not to lung adenocarcinoma, squamous cell lung carcinoma, large-cell lung carcinoma, leukemia, or liver cancer cells (Chen *et al.*, 2008b). In tests with SCLC tissue from a patient and with SCLC cells suspended in blood, the improved and selective binding of the aptamers was observed. Fluorescent and magnetic nanoparticles bound to the SCLC-targeting aptamers could isolate and detect SCLC using magnetism and fluorescence. This suggests that aptamer probes developed from cell-SELEX have the potential for the recognition, extraction, and therapy of selective types of lung tumors and other kinds of cancers.

In addition to the efforts for finding new lung cancer targeting moieties, lung-specific aerosol delivery using conventional tumor-targeting ligands has been used for lung cancer treatment (see Chapter 12 in this book). Gelatin nanoparticles conjugated to avidin and functionalized with biotinylated epidermal growth factor (GP-Av-bEGF) showed targeted internalization in the adenocarcinoma A549 lung cancer cell line compared to the normal lung HFL1 cell line. Furthermore, when GP-Av-bEGF was inhaled into the cancerous lung of a severe combined immunodeficiency (SCID) mouse model, highly efficient selective accumulation of the nanoparticles at the tumor site was confirmed by image quantification (Tseng *et al.*, 2007).

Gene therapy using targeted nanosized carriers for the delivery of an overexpressing vector, shRNA vector, or siRNA for the treatment of lung cancer has been attempted. Hong *et al.* (2012) developed biocompatible glycerol triacrylate-spermine (GT-SPE) polyspermine as a nanosized gene carrier, and showed that the aerosol-delivered GT-SPE/shAKT1 complex successfully suppressed lung tumorigenesis in K-ras^{LA1} lung cancer mice through the suppression of the AKT1 signaling pathway (Hong *et al.*, 2012). Targeted siRNA delivery to the lung was also achieved using cationic polypeptide protamine coated with cationic liposomes containing PEG surface chains (LPD-PEG) (Li and Huang, 2006a, b). In this study, anisamide was used as a ligand for nanocomplex targeting. An *in vitro* study using H1299 lung cancer cells overexpressing the sigma transmembrane receptor showed efficient delivery and increased cytotoxic susceptibility by cisplatin. Furthermore, an *in vivo* study showed that targeted complexes preferentially accumulated at the tumor site (Li and Huang, 2006b).

4.4 Colorectal Cancer

Globally, more than 1 million people develop colorectal cancer every year (Cunningham *et al.*, 2010) resulting in about 0.5 million deaths (Merika *et al.*, 2010). During the most recent 5 years (2005–2009) in the United States, colorectal cancer has been the third most common cancer in women and men, and the third most common cause of cancer death after lung, breast (women), and prostate (men) cancers (Siegel, Naishadham, and Jemal, 2013). Approximately 50% of colorectal cancer patients who undergo surgery develop metastasis to the liver and the lung. Unfortunately, the current therapeutic regimens offer little improvement to the survival of patients with parenchymal metastasis, although the use of the fluorouracil or capecitabine as chemotherapy agents increases the life expectancy (Cunningham *et al.*, 2010).

To treat colorectal cancer, passive targeting has been attempted using polymeric micelles loaded with chemotherapeutic agents (Hamaguchi *et al.*, 2005; Koizumi *et al.*, 2006; Nakajima *et al.*, 2008). 7-Ethyl-10-hydroxycamptothecin (SN-38) loaded on 20-nm micelles showed significantly higher cytotoxicity than free SN-38 in colon-cancer cell line. SN-38 is a biologically active metabolite of irinotecan hydrochloride (CPT-11) and it is a water-insoluble drug. The use of micelles as water-soluble drug carriers improved the accumulation of the drug at the tumor site and its therapeutic effect in a xenograft model of HT-29 colorectal cancer cells (Koizumi *et al.*, 2006).

Active targeting using general cancer-specific markers has been conducted for colorectal cancer treatment. Avastin® (Genentech), a humanized anti-VEGF, and Panitumumab (Amgen, Thousand Oaks, CA), a human antibody against the EGFR, were approved as treatment agents for metastatic colorectal cancer in 2004. These US FDA-approved antibodies have stimulated the study of targeted nanocarriers for delivering therapeutic payloads to colorectal cancer. Cetuximab (IMC-C225), a chimeric (mouse–human) monoclonal antibody-conjugated liposome encapsulating anticancer therapeutics, internalized extensively within 92% of the analyzed tumor cells compared to less than 5% in the case of nontargeted liposomes (Mamot *et al.*, 2003). This system also showed promising results in EGFR-overexpressing tumor models (Mamot *et al.*, 2005).

In 2006, Waldman *et al.* have suggested a novel targeting approach for the treatment of localized and metastatic colorectal cancers. Guanylyl cyclase C receptors, which are overexpressed in metastatic colorectal cancer cells and mediate diarrhea induced by bacterial heat-stable enterotoxins (STs), were used as a target for colorectal cancer treatment. The investigators proposed the conjugation of ST with gold nanoshells, which can undergo resonance excitation by NIR light and emit heat. By doing so, targeted cancer cells were thermally ablated at more than 40 °C (Fortina *et al.*, 2007; Waldman *et al.*, 2006).

In addition to guanylyl cyclase C receptor, Tf receptors 1 (TfR1s, also known as *CD71s*) were also studied as target molecules for colorectal cancer treatment (Table 3). TfR1s are highly expressed in colorectal carcinoma samples of Dukes A or B grade (Stages I and II), and in well-differentiated colorectal carcinoma cells, whereas Dukes C or D grade (Stages III and IV) carcinoma samples or poorly differentiated cells show weak or no expression of TfR1 (Prutki *et al.*, 2006). Linear and star-like hydroxypropyl methacrylamide polymers conjugated with DOX were functionalized with monoclonal antibodies specific to TfR1 (Jelinkova *et al.*, 2003). *In vivo* studies of human colorectal carcinoma SW 620-implanted immunodeficient nu/nu mice xenograft model showed a significantly better therapeutic efficacy in targeted conjugate configurations than free DOX. As star-like targeted conjugates delayed tumor growth significantly longer than linear targeted conjugates, that approach also showed the importance of carrier design on the therapeutic efficiency (Jelinkova *et al.*, 2003).

For the discovery of colorectal cancer-specific targeting moieties, a research group at the University of Utah used the phage display technique to establish peptide libraries that recognized HT29 and HCT116 cells in poorly differentiated and well-differentiated colon carcinomas, respectively (Kelly and Jones, 2003). The peptide discovered in that study was found to bind specifically to tumor tissues derived from colorectal cancer patients. The authors demonstrated the potential use of the peptide in targeted drug delivery system.

4.5 Liver Cancer

Liver cancer incidences and death rates of liver cancer are not as high as other major cancers; however,

they are twice as high in Asian Americans/Pacific Islanders than in White Americans, reflecting the increased prevalence of chronic infection by hepatitis B virus in these populations (Parkin, 2006). As demonstrated by Kim and Kim (2003), asialoglycoprotein receptor (ASGP-R, galactose receptor) is a promising receptor for liver targeting (Kim and Kim, 2003). The authors synthesized biotinylated PEG conjugated with lactobionic acid containing a galactose moiety that exhibits the release of anticancer drug (all-trans-retinoic acid) at a mostly constant rate for over 1 month *in vitro*.

5 COMMERCIALIZED NANOPARTICLES FOR CANCER TREATMENT

Very few nanoparticle-based agents have been approved by the FDA, and most of the commercialized formulations for cancer treatment are based on liposomes (Table 4). Doxil[®], DaunoXome[™], Myocet[®], and L-Annamycine[®] are liposome-based anti-cancer drug formulations for ovarian cancer, Kaposi sarcoma, metastatic breast cancer, and AML, respectively. Abraxane[®] is an aluminum-based nanoparticle approved by FDA for metastatic breast cancer (Gradishar *et al.*, 2005; Moreno-Aspitia and Perez, 2005; Sparreboom *et al.*, 2005). Doxil[®] acts through the sustained-release of DOX resulting in significantly improved efficacy compared to free DOX (Martin, 1998; Park, 2002; Nishiyama and Kataoka, 2006). Abraxane[®] mitigates the hypersensitivity reaction associated with the traditional PTX solvent Cremophor EL. A list of other nanoparticle-based formulations approved by the FDA or those currently in clinical trials is presented in Table 4.

6 PERSPECTIVES OF NANOMEDICINE IN CANCER TREATMENT

Cancer, a life-threatening disease causing nearly 7 million deaths annually worldwide, is still growing. As it is well known, cancer chemotherapy causes severe side effects and multidrug resistance because chemotherapeutics consist of agents with immense cell-killing potential and their mechanism of action is incapable of distinguishing

Table 4. Nanoparticle formulations currently in development or commercially available.

Product	Company	Drug	Formulation	ROA	Application	Status
Abraxane	Abraxis Bioscience, AstraZeneca	Paclitaxel	Albumin-bound nanoparticles	iv	Metastatic breast cancer	In market
DaunoXome™	NeXstar Pharmaceutica	Daunorubicin citrate	Liposome	iv	Kaposi sarcoma in AIDS patients	In market
Emend®	Merck/Elan	Aprepitant, MK869	Nanocrystal® particles	Oral	Chemotherapy-related nausea and vomiting	In market
Myocet® Doxil™ (USA)	Cephalon, Inc.	Doxorubicin	Liposome	iv	Metastatic breast cancer	In market
-Also called Caelyx® (outside USA)	Janssen (a subsidiary of Johnson & Johnson)	Doxorubicin	PEGylated liposome	iv	Kaposi sarcoma, metastatic breast cancer, and ovarian cancer	In market
DaunoXome™	NeXstar Pharmaceutica	Daunorubicin	Citrate liposome	iv	Kaposi sarcoma	In market
L-Annamycin	Callisto Pharmaceuticals	Annamycin	Liposome	iv	Children and young adults with refractory or relapsed ALL or AML	In market
SLIT™ Amikacin	Transave, Inc.	Cisplatin	Liposome	Aerosol	Lung cancer and pulmonary metastases	Phase I/II
N/A	North Central Cancer Treatment Group	Paclitaxel, carboplatin, temozolomide, bevacizumab	Albumin nanoparticles	iv	Stage IV malignant melanoma that is refractory to surgical removal	Phase II
Genexol-PM	Samyang Pharmaceuticals	Paclitaxel	Methoxy PEG-PLA	iv	Breast and lung cancers	Phase II
CALAA-01	Calando Pharmaceuticals	Anti-R2 siRNA	Cyclodextrin-containing polymer (CAL101) and targeting agent (AD-PEG-Tf)	iv	Solid tumors that are refractory to standard-of-care	Phase II
Rexin-G	Epeius Biotechnologies	Dominant negative cyclin G1 construct	Pathotropic nanoparticles	iv	Recurrent or metastatic breast cancer	Phase I
BikDD Nanoparticle	M.D. Anderson Cancer Center/NCI	Pro-apoptotic Bik gene (BikDD)	Liposome	iv	Pancreatic cancer	Phase I/II
Docetaxel-PNP	Samyang	Docetaxel	Polymeric nanoparticles	iv	Advanced solid malignancies	Phase I
NL CPT-11	University of California, San Francisco	CPT-11	Liposome	iv	Recurrent high-grade gliomas	Phase I
(Zhou <i>et al.</i> , 2009)	Individual researchers	Mitoxantrone	PBCA-NPs	iv	HCC	Phase I
(Olavarria <i>et al.</i> , 2000)	Individual researchers	Busulfan	DMPC and DLPC	iv	Chronic myeloid leukemia	Phase II
(Davis <i>et al.</i> , 2010)	Individual researchers	siRNA	Cyclodextrin-containing linear polymer, decorated with PEG and transferrin	iv	Solid melanoma tumors	Phase I/II

(continued overleaf)

Table 4. (Continued)

Product	Company	Drug	Formulation	ROA	Application	Status
(Gagnadoux <i>et al.</i> , 2008)	Individual researchers	9-Nitrocampothecin and Cisplatin, Doxorubicin	Liposome	Aerosol	Primary and metastatic lung cancer	Phase I
(Low <i>et al.</i> , 2007)	Individual researchers	Hapten	Folate-hapten conjugate	iv	Liver cancer	Phase I
(Chien <i>et al.</i> , 2009)	Individual researchers	Paclitaxel, Lapatinib	Albumin-bound nanoparticles	iv	Advanced solid malignancies	Phase II
(Robidoux <i>et al.</i> , 2010)	Individual researchers	5-Fluorouracil/epirubicin/cyclophosphamide	Albumin-bound nanoparticles	iv	Breast cancer	Phase I
(Roy <i>et al.</i> , 2009, Schleicher <i>et al.</i> , 2010)	Individual researchers	Gemcitabine	Albumin-bound nanoparticles	iv	Nonsmall cell lung cancer and small cell lung cancer; metastatic breast cancer	Phase II
(Teneriello <i>et al.</i> , 2009)	Individual researchers	Gemcitabine	Albumin-bound nanoparticles	iv	Epithelial cancer of the ovary, fallopian tube, and peritoneum	Phase I; Phase II
(Conlin <i>et al.</i> , 2010)	Individual researchers	Carboplatin/trastuzumab	Albumin-bound nanoparticles	iv	HER-2 overexpressing metastatic breast cancer	Phase II
PAXEED® Angiotech Pharmaceuticals, Inc.	Angiotech	Paclitaxel	PEO-b-PDLLA	iv.	Multiple sclerosis	Phase II
NK-105 (Hamaguchi <i>et al.</i> , 2005)	Nippon Kayaku Co., Japan	Paclitaxel	PEO-b-PPBA	iv	Breast cancer	Phase I/II
NK-911 (Matsumura <i>et al.</i> , 2004)	Nippon Kayaku Co., Japan	Doxorubicin	PEO-b-P (Asp)-DOX	iv	Various solid cancer	Phase I
SP-1049C (Danson <i>et al.</i> , 2004)	Supratek Pharma, Inc., Canada	Doxorubicin	PEO-b-PPO-b-PEO	iv	Adenocarcinoma of the esophagus and gastroesophageal junction	Phase II

ROA, route of administration; iv, intravenous; im, intramuscular; ALL, acute lymphocytic leukemia; AML, acute myelogenous leukemia; PEG-PLA, poly(ethylene glycol)-poly(lactide); Tf, human transferrin protein; PBCA-NPs, polybutylcyanoacrylate nanoparticles; HCC, hepatocellular carcinoma; DMPC, dimyristoylphosphatidylcholine; DLPC, dilauroylphosphatidylcholine; PEO, poly(ethylene oxide); P(Asp), poly(aspartic acid); PDLLA, poly(D,L lactide); PPBA, poly(4-phenyl-1-butanoate) l-aspartamide. Modified from (Zhang *et al.*, 2008b).

between malignant and healthy cells. Consequently, targeted drug delivery system is highly desirable to minimize the side effects of many chemotherapeutics that have been a fundamental cornerstone in cancer treatment. Although the emergence of new concepts of therapeutics, including therapeutic antibodies, siRNAs, and therapeutics for cancer-specific dysregulated pathways, are recently being explored in clinics, the combined use of conventional chemotherapeutics is still indispensable for patient survival. Furthermore, because

site-specific delivery remains a major hindrance to efficacious therapy, the cancer patient survival rate is still modest.

Over the last decades, extensive attempts have been made to overcome the obstacles associated with conventional chemotherapy via different strategies, thereby enhancing the life expectancy of cancer patients. Innovative strategies have been developed for imaging, targeting, and therapeutic cure using nanocarriers such as colloidal gold, magnetic, and pH-sensitive nanoparticles that have been

discussed in this chapter. Such a multistage drug delivery platform has been engineered to release the incorporated drug under “special” conditions of the tumor environment by altering the pharmacokinetics of the therapeutics. However, their selectivity and effectiveness remain to be optimized. To improve this, it is necessary to extensively explore specific surface target molecules and to deeply study cancer genomics to identify culprit genes for specific cancer types, in addition to developing methods for site-specific and controlled-release delivery carriers. Insights into biomarkers and rational design of the carriers will allow clinicians to tailor drug regimens to specific tumors, offering rationally designed therapies of a more personalized nature. Combining one or more advanced approaches of nanomedicine will serve new paths for safe, specific, and efficient cancer treatment.

7 CONCLUSIONS

The chapter described nanotechnology-based tumor treatment approaches for the improved delivery of already established chemotherapeutics. Nanotechnology has been an extremely hot topic over the last decade, and the field of medicine, specifically cancer treatment, is not an exception. As big strides have been made in the material science arena, more effective delivery of therapeutics using nanoparticles has resulted in the development of novel methods to treat and diagnose cancer. However, there are still obstacles in the application of nanoparticles in the biomedical field to the fulfillment of the expectations. Very few nanoparticle-based agents are commercialized or in clinical testing for cancer diagnosis or treatment. Efforts for reducing the toxicity of nanomaterials, identifying biocompatible materials, and searching for molecular targets will advance the ability to improve the delivery of drugs at the tumor level while decreasing the toxicity to normal tissues. Although there is still a long way to go before nanotechnology can truly benefit patients with cancer, continuous research will help achieve this goal as many have hoped it would.

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Nanomedicine in Diabetes: Using Nanotechnology in Prevention and Management of Diabetes Mellitus

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1 INTRODUCTION

Diabetes mellitus (Greek *diabētēs*: passing through, Latin *mellitus*: honey-sweet) is a condition characterized by an elevated blood glucose (hyperglycemia). It is not a single disease; rather, it is a group of metabolic disorders with hyperglycemia as a common denominator. The costs associated with diabetes in the United States were >240 billion dollars in 2012, with an average of \$13 700/patient (ADA, 2013a). By comparison, the annual budget for the National Institute of Diabetes and Digestive and Kidney (NIDDK) Diseases, a key branch of National Institute of Health dedicated to research in diabetes, was just under 2 billion dollars in the same year (NIH, 2013a). These data allow us to begin to appreciate how dysregulation of a simple sugar (Figure 1) can have serious consequences. This is further illustrated by the statistics compiled by the Centers for Disease Control and Prevention in the 2011 National Diabetes Facts Sheet (CDC, 2011) (Box 11.1).

BOX 11.1: DIABETES IN THE UNITED STATES—2011 NATIONAL DIABETES FACTS SHEET.

Key Statistics

25.8 million people affected
7 million (27%) undiagnosed
Seventh leading cause of death

Major Complications

Heart disease and stroke
Kidney disease
Lower limb amputations
Blindness

Patients on Treatment

16% no medication
84% on treatment

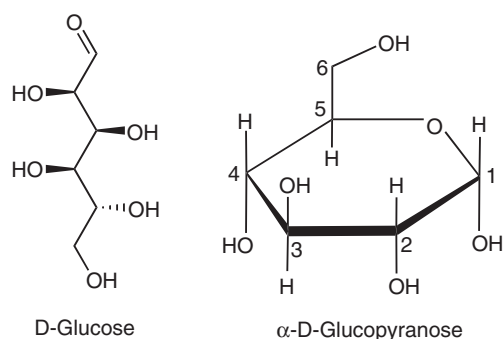


Figure 1. Commonly used structural formulas depicting glucose. Glucose comes from the Greek word *glukus* (sweet), which describes the physical property of taste. Synonym dextrose is related to optical property of glucose in the solution—when a plane-polarized light is passed through a solution of glucose, glucose rotates to the right making it dextrorotary. D-Glucopyranose, another synonym for glucose, encompasses two properties of the molecule in its name—the stereoisomerism (D,L) and ring structure of thermodynamically favored cyclical six-member structure similar to pyran ring.

Considering a large number of affected people and the high prevalence of clinically significant complications, diabetes-related research has been steadily increasing over the last few decades (Figure 2). In health sciences, the number of diabetes-related publications increased more than five times in the last decade alone (~22 000 publications in 2012 versus ~4000 publications in 1992; Figure 2a). In the same period, nano publications in health sciences remained <1000 (Figure 2a), although they expanded significantly in the subject area of physical sciences (Figure 2b). An increase in diabetes and nano research in both health and physical science subject areas is suggestive of the interdisciplinary, collaborative nature of research (Figure 2). Collaborative research is a *condicio sine qua non* in developing novel treatments and diagnostics. Excellent, and current, example is the application of mathematics to big data analyses such as in genetics and genomic sciences. This field requires teams of scientists specialized in systems biology and mathematical modeling, geneticists, biologists, pharmacologists, and physicians working together in order to uncover biologically meaningful and clinically relevant connections. Perusal of the literature uncovered an increase in studies using mathematical modeling in health sciences, as well as gene microarray studies (Figure 2).

2 NANODIAGNOSTICS IN DIABETES—BLOOD GLUCOSE MONITORING

An early detection of blood glucose abnormalities is essential for the diagnosis and successful intervention in diabetes. The two most common types of diabetes mellitus are type 1 (T1DM) and type 2 (T2DM). Most T1DM patients have autoimmune destruction of insulin-producing β cells in the islets of Langerhans, and become dependent on exogenous insulin for survival. Some T1DM patients have idiopathic disease with fluctuating absolute dependence on insulin (ADA, 2013b; Herold *et al.*, 2013). T2DM patients may go undiagnosed for years, starting with insulin resistance that gradually leads to frank diabetes. In terms of percentage of afflicted patients, >90% are T2DM and up to 10% are T1DM. The impairment of glucose metabolism and insulin sensitivity can, unfortunately, already be seen in obese children and young adults (Sinha *et al.*, 2002) along with the evidence of metabolic syndrome and increased risk for comorbidities such as cardiovascular disease (Weiss *et al.*, 2004). Box 11.2 summarizes some of the clinical parameters used to establish a diagnosis of diabetes (ADA, 2013b). In both T1DM and T2DM, managing blood glucose is critical.

BOX 11.2: SOME OF THE TESTING CRITERIA FOR DIAGNOSING DIABETES.

Glycated Hemoglobin

≥6.5%; diabetes.
5.7–6.4%; prediabetes.
<5.7; normal.

Fasting Blood Glucose

≥126* mg dl⁻¹; diabetes
100–125 mg dl⁻¹; prediabetes
<100 mg dl⁻¹; normal
*On two independent occasions.

Random Blood Glucose

≥200 mg dl⁻¹; diabetes
140–199 mg dl⁻¹; pre-diabetes
<140 mg dl⁻¹; normal

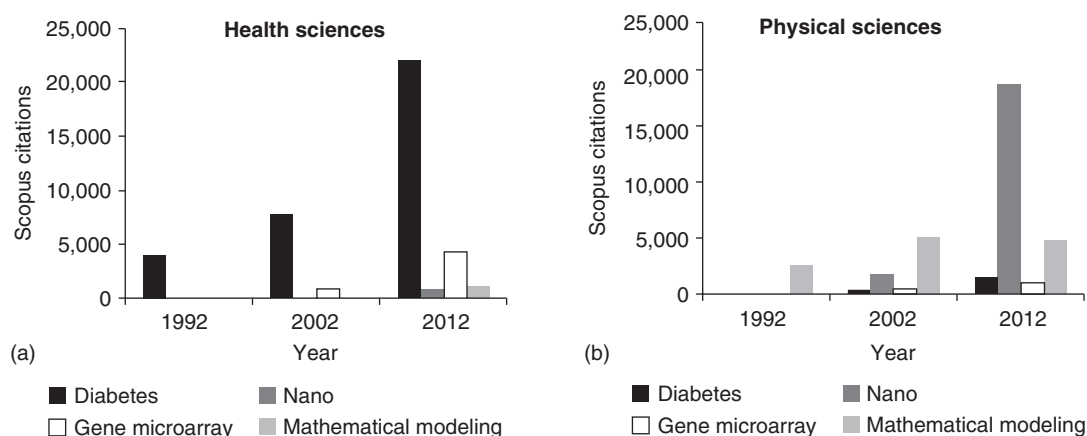


Figure 2. Publication trends in diabetes and nano research in health and physical sciences. (a) In health sciences, diabetes research has seen a steady increase between the years 1992, 2002, and 2012 (4056, 7849, and 22 031 publications). By comparison, nano research in health sciences had just started gaining momentum (0, 45, and 856 publications). Conversely, studies using gene microarrays and mathematical modeling have increased in the same period of time—0, 886, and 4257 and 125, 299, and 1124 publications, respectively. (b) In physical sciences, almost an exact opposite is seen; 76, 1733, and 19 275 publications in nano and 133, 331, and 1419 in diabetes. Publications using mathematical modeling were more or less steady 2536, 5210, and 4882 and gene microarrays were 0, 351, and 965. The key observation in these graphs is the increasing number of nano publications in health sciences and of diabetes publications in physical sciences. This suggests the increasingly interdisciplinary nature of research in recent years. Note: Scopus database (www.scopus.com) was perused on 17 May 2013 using the keywords indicated in the graph legends along with the following search parameter limits: Search in: “article title, abstract, and keywords”; Limit to: Date range: “1992–1992”, “2002–2002”, and “2012–2012”; Document type: “article”; Subject areas: “Health sciences” (a) and “Physical Sciences” (b).

In order to adequately manage blood glucose, its concentration must be determined accurately, reproducibly, and with great sensitivity. The most common approach to detect abnormal blood glucose is using a glucometer. The importance of daily self-monitoring of blood glucose by patients themselves has been recognized in the 1970s (Sonksen, Judd, and Lowy, 1978). Today, finger prick measurements of blood glucose are part of a daily routine for many diabetic patients. The procedure is performed by a hand-held device using disposable glucose strips, and the glucose detection is based on an enzyme-mediated electrochemical measurement (Figure 3). Most commonly, the enzymes are glucose dehydrogenase or glucose oxidase, whereas coenzymes may include flavin adenine dinucleotide, nicotinamide adenine dinucleotide, or pyrroloquinolinequinine (Heinemann, 2010).

Nanotechnology aspect to glucose measurement includes the investigation of nanoelectrodes with high surface area for nonenzymatic oxidation of glucose. For example, the use of carbon nanotubes in nanosensors is largely due to their ability to promote electron-transfer reactions and resist surface fouling.

The unique feature of the atomic structure of carbon nanotubes allows for their metal or semiconductor properties, which differs with the wrapping angle of the sheet(s) of graphite (Wilder *et al.*, 1998). In a recent example of constructing a glucose-sensing nonenzymatic electrode, carbon nanotubes (~80 nm in diameter) were grown vertically on Si/SiO₂ substrate creating carbon “forests” with a density of 3.5×10^{-9} carbon nanotubes $\times \text{cm}^{-2}$. The nanotubes were coated with Ni nanoparticles (~15 nm in diameter) to create a carbon-nanotube/Ni electrode. Following the exposure to glucose, Ni(III) oxidizes glucose, producing Ni(II), and the resulting change in the number of Ni species is registered as a change in the peak currents on the anode and cathode (Zhu *et al.*, 2013). Importantly, the system was tested in five clinical blood samples and the measured glucose was within ± 2.8 –4.7% of that obtained in the hospital. These data support the proof of principle of the system in detecting glucose in real life samples where nonglucose blood components are abundant.

Glucose need not only be analyzed in blood. Development of unorthodox noninvasive ocular

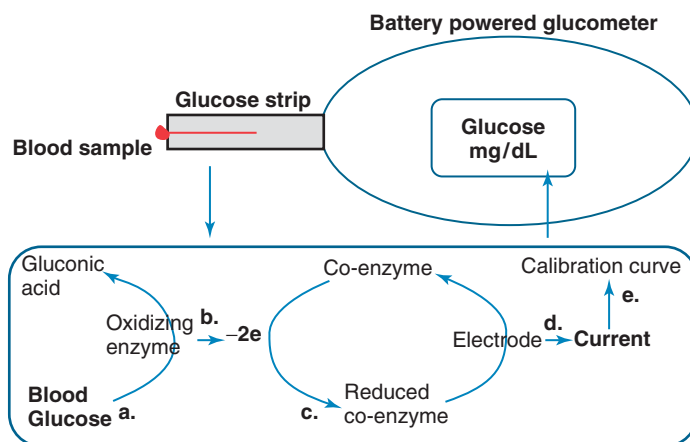


Figure 3. Basic procedures and principles of self-monitored blood glucose measurement. Disposable glucose strip that contains the necessary electrodes, reaction enzymes, and chemicals is inserted in the battery-powered glucometer, which turns the glucometer on. Blood sample, for example, from a finger prick, is allowed to fill the glucose strip through capillary action. Within 5–30 s, the reading of glucose is displayed on the glucometer (Glucose $\text{mg dL}^{-1} \rightarrow$ fasting blood glucose concentration $<5.6 \text{ mmol L}^{-1}$ is considered physiologically normal, as is a blood glucose concentration of 7.8 mmol L^{-1} 2 h after an oral glucose tolerance test). The measurement is an electrochemical reaction, which, in general, involves the following steps: (a) glucose oxidizing enzyme is reduced in presence of blood glucose; (b) mediator (coenzyme) is reduced thus recovering the glucose-oxidizing enzyme to its nonreduced form; (c) voltage is applied between the working and the reference electrodes and the reduced coenzyme is oxidized; (d) the resulting current between the working and the reference electrodes is measured and used to calculate and display the glucose (glucose is proportional to the current) (e).

blood glucose-monitoring sensors has gained traction in recent years. Bodily fluids such as tears have been shown to reflect the difference in glucose levels between nondiabetic and diabetic persons. In a study published in the 1950s, 101/119 diabetics (85%) with elevated blood glucose also had a positive lachrymal fluid glucose test (Lewis, 1958). Nanotechnology has taken on development of contact lenses that are capable of detecting glucose in lachrymal fluid. One such example includes fluorescence-based lens sensors utilizing fluorescent mesoporous silica nanoparticles ($\sim 50 \text{ nm}$) with incorporated organic dyes (Zhang *et al.*, 2011). The general principle of glucose detection is based on quantifying a change in fluorescence resonance energy transfer by displacement of one of the organic dyes by glucose, which has been investigated under controlled conditions using laser scanning confocal microscopy, and fluorescence measurements (peaks detected at 510–520 nm, and 570–580 nm) (Zhang *et al.*, 2011). In terms of clinical applicability a contact lens that could change color in the visible spectrum, in response to significant deviations of glucose (increase/decrease), would presumably offer a useful alternative to patients, especially those that may already be using contact lenses and would not be averse to having a contact lens in

the eyes. The authors conclude their study with “A new design has been developed to overcome the shortcoming,” suggesting that some modifications may have already been attempted or realized (Zhang *et al.*, 2011). A comprehensive overview of optical methods for sensing glucose is available (Steiner, Duerkop, and Wolfbeis, 2011).

Electrochemical-based glucose sensors require power, which is why development of a self-powering nanotechnological product that uses ascorbate from lachrymal fluid to provide power may prove useful in glucose-sensing contact lenses (Falk *et al.*, 2013). The system is composed of a cathode modified with bilirubin oxidase and gold nanoparticles, and an anode modified with gold nanoparticles and tetrathiafulvalene and tetracyanoquinodimethane as catalysts of ascorbate oxidation. However, the contact lenses have the potential for serious side effects in diabetic patients whose eyes are compromised by the disease itself, and these must be considered (O'Donnell and Efron, 2012). Still, it is very likely that a population of patients that can wear corrective contact lenses without significant complications may benefit from lenses' glucose-sensing aspect. Although currently not considered as the first line approach to glucose measurement, additional

advances in development of glucose-sensing contact lenses with/without vision correction features represent a possible alternative to be used under restricted conditions.

Some of the challenges with glucose nanosensors, as discussed by Cash and Clark, include the need for systematic comparison with commercial sensors to demonstrate cost/benefit ratios that would justify/favor the use of for example, nanoelectrodes (Cash and Clark, 2010). Considering that the largest expenditure for diabetic patients using glucose strip sensors are the strips themselves (one-time use, disposable), developing systems that could provide repeated glucose measurements could be one of the approaches of advancing the new technologies of glucose detection. This brings us to the topic of continuous glucose monitors that are discussed under the section of an artificial pancreas development further in this chapter.

Not all technologies seeking application in diabetes are based on glucose monitoring. There are approaches to monitor/diagnose diabetes, which are in preclinical development, that rely on analyses of air samples. “Breathalyzer” for diabetics has completed the proof of concept stage (Righettoni, Tricoli, and Pratsinis, 2010). The aluminum oxide substrate with gold electrodes was deposited with tungsten oxide nanoparticles (~12 nm) for analyses of acetone in air samples (Righettoni, Tricoli, and Pratsinis, 2010). The authors suggest that the system could be used as a breath analyzer in diabetes. Indeed, acetone build up and a characteristic breath odor can be present in diabetic patients as a warning sign of a potentially life-threatening condition—ketoacidosis. For a historical perspective on discovery and establishment of acetone in diabetes, please refer to (Crofford *et al.*, 1977). The detection process discussed in the paper by Righettoni, Tricoli, and Pratsinis (2010) included an oven with temperatures up to 500 °C and a mass spectrometer for analyses of the exhaust flow. The authors demonstrated in principle that physiologically relevant changes in acetone concentrations can be measured under controlled conditions even in the presence of variable humidity such as would be found in human breath. Such a proof of principle makes it quite interesting to learn how such detection systems may be further developed in terms of, for example, variability, portability, and potential clinical use.

Importantly, in diabetes management, it is not simply the technology or hand-held device that is important in managing the disease. Significant role is played by the patients and the providers. There is a strong evidence from the literature, coming from the analyses of >100 systematic reviews, which suggest that patient education, provider role change, and telemedicine can improve management of blood glucose and cardiovascular complications of diabetes (Worswick *et al.*, 2013).

3 NANOTECHNOLOGY IN GENOMICS AND POTENTIAL USE IN DIABETES DETECTION/PREVENTION

Systems for DNA sequencing continue to be applied to various human samples in order to obtain large amounts of data, sifting through which is expected to unravel novel details about the pathology of the diseases and, consequently, allow novel diagnostics, prognostics, and therapeutic targets to be discovered. Nanotechnology plays a critical role in many of these sequencing systems.

For example, single molecule real time sequencing system manufactured by Pacific Biosciences is one of the latest next generation sequencing platforms. Their system utilizes consumable substrates that contain 150 000 of 70 nm wide zero-mode waveguides. Zero-mode waveguides are nano-optic structures that are subwavelength holes in a metal film (Levene *et al.*, 2003). The laser excitation coming from below these subwavelength nanosized holes cannot pass through, rather it penetrates the lower end of the waveguide allowing for a detection volume of 20×10^{-21} l (zeptoliter). This allows for the detection of DNA polymerase activity in the presence of micromolar concentrations of fluorescent-labeled nucleotides with minimal background (Korlach *et al.*, 2008; Levene *et al.*, 2003). The arrays of zero-mode waveguides, each with a single DNA polymerase–template complex at the bottom, allow for simultaneous detection of tens of thousands of continuous base incorporations (Biosciences, 2013a, 2013b).

Another set among the next generation sequencing platforms (Mardis, 2013) uses changes in electrical current to monitor nucleotide incorporation during sequencing. NanoPore Technologies®, as the name suggests, bases its detection technology on nanosized protein pores embedded in a synthetic

membrane through which charged molecules are driven by electrophoresis. Dimensions of the transmembrane part of the α -hemolysin protein pore (the barrel) are ~ 2.6 nm width and ~ 5 nm length. Thousands of such nanopores can be scaled onto a chip and consumable cartridges for thousands of parallel reactions (Nanopore, 2013). The principles of base detecting technology used in the nanopore sequencing system include construction of a nanopore using α -hemolysin protein with N139Q mutation and covalent attachment of aminocyclodextrin at L135C cysteine residue, which constricts the channel and allows continuous nucleoside monophosphate detection without fluorescent labeling at high rates of translocation through the pore (Clarke *et al.*, 2009). The individual changes in the current are registered as clearly distinguishable peaks of residual pore current, for example, dGMP ~ 29 pA, dTMP ~ 31.5 pA, dAMP ~ 34.5 pA, dCMP ~ 42 pA, and methylated dCMP at ~ 33 pA. Integrating the exonuclease enzyme that cleaves and passes the nucleotides to the nanopore, along with the above-described nucleotide detection technology is utilized by NanoPore Technologies[®] (Clarke *et al.*, 2009; Nanopore, 2013). The details of the basic principles of different variations of nanopore technology are reviewed in the paper by Venkatesan and Bashir (2011).

Sequencing technologies such as those discussed here can advance the prevention and diagnosis of diabetes through fast, accurate, and affordable screening of persons at risk. However, we are not yet at the stage where this is clinically applicable globally given the current costs of the whole genome sequencing, large numbers of people at risk for diabetes, and the unresolved legal and ethical considerations surrounding the genetic testing. In terms of cost, the price of whole genome sequencing has dropped by a factor of $>12\,000$ in a period from September 2001 (\$95 263 072) and January 2012 (\$7666) (Wetterstrand, 2010); since January 2012, costs have remained around \$6000 (Wetterstrand, 2010). On the other hand, costs of diagnosing, for example, T2DM, according to a retrospective study conducted in the United Kingdom, averaged GBP 377 for 5720 screenings done on 2763 patients during a period of 3 years (Pereira Gray *et al.*, 2012). During that time, 54 patients (1.95%) were diagnosed with diabetes by the retrospective screening. Study utilized the data from medical records

of patients that were seen in the clinic for conditions that are considered risk factors for diabetes (e.g., hyperlipidemia, obesity, and cardiovascular disease) who were offered to have their blood glucose checked as well. In these retrospective analyses, patients with existing T1DM or T2DM, as well as pregnant women, were excluded from the analyses (Pereira Gray *et al.*, 2012). Modeling the costs of diagnosing diabetes using parameters, such as, age-grouping, different known risk factors and similar, is available and includes estimates in a similar range as (Pereira Gray *et al.*, 2012), namely falling within GBP 500–900 (Waugh *et al.*, 2007). The readers are referred to (Waugh *et al.*, 2007) for exact details of the analyses and models, which are too great to recapitulate here. Considering such analyses, and as already mentioned, the price of whole genome sequencing should still come down from the current $\sim$ \$6000 mark to make it economically feasible. On the other hand, when considering the tests, it is important to consider not only the cost of a test itself but the likelihood of successfully detecting prediabetes in at-risk populations as well (Chatterjee *et al.*, 2010; Zhang *et al.*, 2003). The costs of diabetes screening today generally run in the range of few hundred dollars (Phillips *et al.*, 2009). While developing standard procedures that will allow the use of genetic and genomic approaches to be used in screening patients at risk for diabetes is very appealing, their cost/ratio benefit remains to be established.

From the legal standpoint, the rights of the persons who undergo genetic testing are protected with respect to health insurance and employment in the United States by the “The Genetic Information Nondiscrimination Act of 2008” (EEOC, 2008) although many ethical, legal, and social issues remain (Knoppers, Zawati, and Kirby, 2012). Moreover, not everyone is convinced that genetic information should be afforded status different from other medical—that is, confidential—information (Khoury, Evans, and Burke, 2010). Debates also continue about the seeming discrepancies between the “traditional” hypothesis-driven research and large-data collection research driven by genetics and genomic sciences (Golub, 2010; Schadt and Bjorkegren, 2012; Weinberg, 2010). Logic would dictate that, with time, narrowing down the number of candidate hypotheses by utilizing systems

biology will be accepted as complimentary to “traditional” research approaches of validating select hypotheses, and vice versa.

Nonetheless, genome-wide association studies and meta-analyses of published data have resulted in identification of tens of different T2DM susceptibility variants not all of which are the same between geographically different populations, for example, Europeans and Asians (Kooner *et al.*, 2011; Morris *et al.*, 2012). An excellent review summarizes some of the key differences between Eastern and Western populations (Kong *et al.*, 2013). For more detailed information on the subject, readers are referred to the following reviews and references there in (McCarthy, 2010; Palomaki *et al.*, 2013).

4 EXAMPLES OF NANOMEDICINES IN DIABETES

With the exception of insulin, very few drug formulations have been investigated for potential treatment of diabetes using nanoscience/nanotechnology. As the insulin delivery has been extensively covered, the reader is referred to some of the recent reviews and articles as a starting point in perusing this subject (Danne and Bolinder, 2013; Gu *et al.*, 2013a; Heinemann, 2013; Shimkunas *et al.*, 2009; Sonia and Sharma, 2012). It has been intentionally omitted here, and priority has been given to other nanomedicines.

Presently, one adjunct drug treatment—fenofibrate—for prevention of cardiovascular events in diabetics (Rosenblit, 2012; Tonkin *et al.*, 2012), is available on the market in two nanocrystalline formulations (FDA, 2013a, 2013b). Fenofibrate is a poorly water-soluble drug, with an estimated octanol water partition coefficient $\log K_{o/w}$ of 5.19, and water solubility of <1 mg per liter (0.42 mg l^{-1}) (PubChem, 2013). Poorly water-soluble drugs have limited bioavailability because of, for example, poor dissolution and absorption in the gastrointestinal tract. Nanonization dramatically increases the surface area of the drug and leads to the establishment of higher drug concentration gradient between the gut and the blood, via increased saturation solubility and dissolution velocity, allowing for improved absorption of drug during its passage through the gut (Gao *et al.*, 2012; Merisko-Liversidge and Liversidge, 2011). The general principle of surface

area increase through nanonization is illustrated in Figure 4.

Proof of principle study using an oral formulation of glucagon-like peptide 1 analog, exendin-4, demonstrated *in vivo* potential for oral administration of exendin-4 (Ahn *et al.*, 2013), which is normally injected sub-cutaneously as an adjunct treatment for T2DM (FDA, 2011). Low molecular weight chitosan was used to conjugate the cystein-modified exendin-4 and formulate the nanoparticles of ~ 120 nm in diameter. Following oral administration of exendin-4 nanoparticles, significant decrease in blood glucose was observed during the glucose tolerance test with both 4 and $40 \mu\text{g kg}^{-1}$ dose of exendin-4; exendin-4 ($40 \mu\text{g kg}^{-1}$) without nanoparticulate formulation had no effect on reducing blood glucose (Ahn *et al.*, 2013). The bioavailability of nanoformulated exendin-4 following $400 \mu\text{g kg}^{-1}$ oral administration was 6.39%. As is the case in diabetes, patients are seldom treated with a single drug. Chuang and colleagues investigated and demonstrated that coadministration of nanoparticles containing exendin-4 ($300 \mu\text{g kg}^{-1}$) and those containing insulin (30 IU kg^{-1}) achieved greater blood glucose-reducing effect, during a glucose challenge in rats with streptozotocin-induced diabetes, than either nanoformulated drug alone (Chuang *et al.*, 2013). The polymer used in coadministration study was chitosan (80 kDa) and poly- γ -glutamic acid (60 kDa), with average drug loaded particle size of ~ 250 nm.

It would appear, however, that glucose-lowering effect of exendin-4 alone in (Chuang *et al.*, 2013) was less than the one observed by (Ahn *et al.*, 2013). For example, 60 min after the 1 g kg^{-1} oral glucose challenge blood glucose in rats treated with nanoformulated exendin-4, contained in enteric coated capsules, was $\sim 183 \text{ mg dl}^{-1}$ versus 217 mg dl^{-1} in rats treated with free form oral insulin and exendin-4 in enteric coated capsules (Chuang *et al.*, 2013); a decrease of 34 mg dl^{-1} or $\sim 16\%$. Conversely, the nanoparticles made using 9 kDa chitosan conjugated with cystein-modified exendin-4 showed that 60 min after 2 g kg^{-1} injection challenge with glucose, blood glucose levels in db/db mice were $\sim 210 \text{ mg dl}^{-1}$ ($40 \mu\text{g kg}^{-1}$) and $\sim 280 \text{ mg dl}^{-1}$ ($4 \mu\text{g kg}^{-1}$) versus 391 mg dl^{-1} in free form of oral exendin-4 (Ahn *et al.*, 2013); this represents a difference of 181 mg dl^{-1} ($\sim 46\%$) and 111 mg dl^{-1}

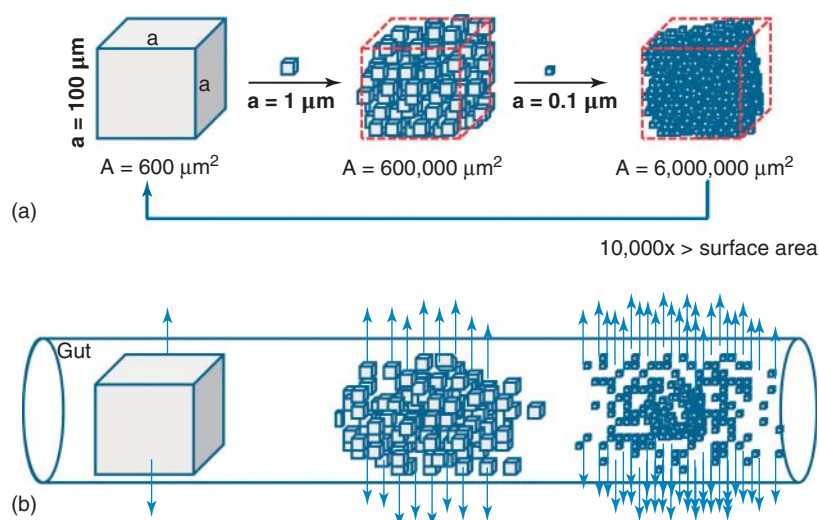


Figure 4. Nanonization drastically increases the surface area. (a) If we were to break an idealized drug particle (cube shaped, $a = 100 \mu\text{m}$, surface area $A = 6 \times a^2 = 600 \mu\text{m}^2$) into smaller cubes ($a = 1 \mu\text{m}$), the resulting surface area would be up to 100× greater than that of a single cube with $a = 100 \mu\text{m}$. Further breaking down the particles of $a = 1 \mu\text{m}$ into cubes with $a = 100 \text{ nm}$ ($0.1 \mu\text{m}$) would increase the total surface area of drug particles by up to 10 000 compared to the starting particle. (b) Steps in (a) would allow greater contact surface area of drug with the gut lumen, leading to establishment of higher drug concentration gradient ($\uparrow$) and eventually greater absorption of poorly water-soluble drugs from gut to blood. Notes: illustration purposes only; not drawn to scale; actual particle shapes and drug yields vary according to manufacturing and nanonization procedure used (Merisko-Liversidge and Liversidge, 2011).

(~28%), respectively. Each study employed different animal models, different formulations of the nanoparticles, and specific glucose tolerance test protocols, all of which preclude any direct comparisons between the two studies. Notwithstanding, looking at the changes in glucose values in treated animals, it would appear that greater success in lowering blood glucose was achieved by conjugated exendin-4 using 9 kDa chitosan.

Different approach to developing a delivery system for exendin-4 focused on designing a nanonetwork of polymers with controlled release of the drug, and administered under the skin (Kim *et al.*, 2013). Kim and colleagues used 20–80 (w/w) solution of 20 wt% water solution of phosphatidylcholine and 35 wt% water solution of Pluronic F-68 tri-block copolymer to prepare nanoparticles, which were then mixed with exenatide and phosphatidylcholine vesicles (liposomes). Next, the vesicle nanoparticles were mixed with Pluronic and freeze dried to form the final product—multilayer nanoparticles that had Pluronic coating stabilizing the phosphatidylcholine vesicles (liposomes). When administered sub-cutaneously as a single injection (10 nm kg^{-1} exenatide) to 6-week-old db/db mice, blood glucose in animals receiving free exenatide

was maximal just under 2 h after the injection ($\sim 20 \text{ mmol l}^{-1} = \sim 360 \text{ mg dl}^{-1}$). At 3 h, the effect of free exenatide was almost completely gone, whereas blood glucose in mice receiving exenatide in a form of multilayer nanoparticles remained low ($\sim 16 \text{ mmol l}^{-1} = \sim 290 \text{ mg dl}^{-1}$) compared to vehicle treated animals ($\sim 32 \text{ mmol l}^{-1} = \sim 580 \text{ mg dl}^{-1}$). At 4 h, the glucose in db/db mice treated with multilayer nanoparticles remained around $\sim 16 \text{ mmol l}^{-1} = 290 \text{ mg dl}^{-1}$, gradually rising to $\sim 22.5 \text{ mmol l}^{-1} = \sim 405 \text{ mg dl}^{-1}$ at 12 h, and remaining largely unaltered at 24 h (Kim *et al.*, 2013). Even longer effect on blood glucose in db/db mice was achieved using inhalable poly(lactic-co-glycolic acid) nanoparticles coated with chitosan and loaded with palmitic acid conjugated exendin-4 (Lee *et al.*, 2013). As discussed earlier, chitosan was used for its mucoadhesive properties (Sogias, Williams, and Khutoryanskiy, 2008), which increases the resident time of particles, in this case, in the lungs (de Jesús Valle *et al.*, 2008; Lee *et al.*, 2013). Administration of nanoparticles containing modified exendin (100 nmol kg^{-1}) led to $\sim 100 \text{ mg dl}^{-1}$ blood glucose in db/db mice at 12 and 24 h after administration, whereas vehicle treated mice remained $>400 \text{ mg dl}^{-1}$. At 2 days, the glucose rose to

$206 \pm 75 \text{ mg dl}^{-1}$, and the effect was lost after 4 days.

5 NANOTECHNOLOGY AND ARTIFICIAL PANCREAS

The problem with most drug delivery systems, including those discussed in previous sections, is that they do not respond to physiological and pathological stimuli in a manner that the endocrine pancreas does. Therefore, a lot of effort is put into development of nanosensors that could not only sense the glucose levels but also dispense the therapeutic agent. Continuous blood glucose monitoring allows for a more insightful perspective into the patients' daily glucose variations, response to treatment and it also paved the way, in part, to development of devices that combine the glucose monitoring with insulin administration. Such medical devices aim to mimic the actions of endocrine pancreas. There are several continuous glucose-monitoring products available in the market to patients by prescription (FDA, 2006, 2007, 2008, 2012). To put it plainly, the components of these devices include a subcutaneous glucose sensor that transmits glucose readings to a wireless monitor worn by the patient. The limiting factors of these sensors are (i) the sensor needs to be replaced within up to a week and (ii) the patients, occasionally, still have to use the finger prick glucose monitoring to confirm the readings because of inherent lower accuracy of the continuous glucose-monitoring devices. On the other hand, the advantages of continuous glucose monitoring include a more detailed clinical insight into the individual patient's glucose fluctuations (readings can be obtained every 1–5 min), analyses of glucose fluctuation patterns, and audio alarms for out of range (high, low) glucose, eventually allowing improvements in glucose management. The “ideal” device would not only sense the glucose, but would adequately dispense insulin. Indeed, combinations of continuous glucose monitors and insulin pumps are available.

For example, recently completed clinical trial examined insulin pumps with an important safety feature—suspending the insulin administration to prevent hypoglycemia (Bergenstal *et al.*, 2013). The study was conducted in T1DM patients with documented episodes of nocturnal hypoglycemia; 121 patients were assigned to treatment

(threshold-suspend device) and 127 to control group. The glucose-sensor-assisted insulin-pump was successful in significantly reducing the incidence of night-time hypoglycemic events by $\sim 32\%$, in this open label, multicenter, randomized, and controlled clinical trial (Bergenstal *et al.*, 2013). As discussed by the authors, some of the limitations of the study may include preselection of patients, and the quality of life improvement was not observed, possibly because of the relatively short 3-month duration of the trial.

Further improvement in nocturnal hypoglycemia can be attained using devices that integrate glucose-sensing and insulin-dispensing activities, with information on disease management pre-entered from patient's records (Phillip *et al.*, 2013). The study by Phillip *et al.* (2013) was much shorter than Bergenstal *et al.* (2013), lasting only 3 days, and aimed to test the efficacy and safety in 56 patients. However, it did involve the cross-over design and elaborate software management of the device, which used not only “yes”/“no” commands for insulin dispensing based on glucose sensing but also “if”/“then” type of decisions in maintenance of normoglycemia based on the fuzzy logic theory (Atlas *et al.*, 2010; Phillip *et al.*, 2013). The details of the development and application of fuzzy logic theory in this device can be found in Atlas *et al.* (2010), and additional clinical trials that are continuing with further evaluation/validation of the device are ongoing (Nimri *et al.*, 2012, 2013a, 2013b).

In order to appreciate some of the complexities in designing the above-discussed medical devices, basic glucose homeostasis is depicted in Figure 5. Elevation of glucose in the blood, for example, after a meal, leads to insulin release from endocrine portion of the pancreas (β -cells in the islets of Langerhans), which promotes (i) glucose storage in the liver as glycogen, (ii) glucose uptake by skeletal muscles, and (iii) lipogenesis in the adipose tissue. Conversely, a drop in blood sugar leads to the release of a different hormone from the endocrine pancreas—glucagon (α -cells in the islets of Langerhans), which leads to a different set of actions in the above-mentioned tissues: (i) degradation of glycogen, glucose generation, and lipogenesis in the liver, (ii) fatty acid uptake in the skeletal muscles, and (iii) lipolysis in the adipose tissues (Cherrington, 1999).

Of these actions, medical devices mimic administration of insulin based on the continuous glucose monitoring—a glucose sensor is usually inserted

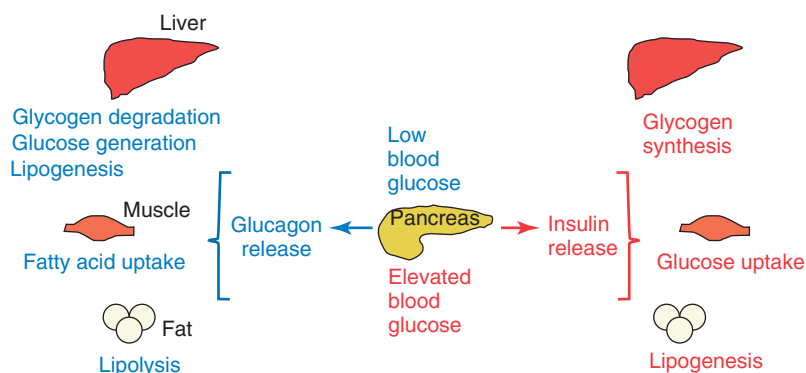


Figure 5. Basic regulation of blood glucose. Right-hand side, red letters, indicates main events in a state of elevated blood glucose: a rise in blood glucose leads to insulin release from the pancreas and clearance of glucose from the blood mainly by the skeletal muscles, the liver, and a small amount by the fat tissue (major effect of insulin in fat is lipogenesis). Left-hand side, blue letters, indicates main events in a state of decreased blood glucose: a drop in blood glucose leads to glucagon release from the pancreas and release of glucose into the blood stream by the liver through degradation of glycogen and glucose synthesis; muscles take up fatty acids and adipose tissue undergoes lipolysis. As glucose is a hydrophilic molecule, its transport across plasma membranes is facilitated by a family of glucose transporters (Thorens and Mueckler, 2010). Once inside the cells, the excess glucose is stored in a polymer form as glycogen; bonds between glucose molecules in $\alpha 1 \rightarrow 4$ position are polymerizing and in $\alpha 1 \rightarrow 6$ position branching (Figure 1, glucopyranose ring). Major site of glycogen storage in the body is skeletal muscle ($\sim 80\%$) (DeFronzo *et al.*, 1981), followed by the liver ($\sim 19\%$ of ingested glucose stored as glycogen in healthy volunteers following a mixed meal) (Taylor *et al.*, 1996). However, one of the main differences between these two glycogen storage sites is that liver glycogen can be broken down to glucose, which can then be released back into the blood stream as needed, whereas muscle glycogen serves mainly as a local energy source (Jensen *et al.*, 2011). The latter is due to the lack of glucose 6-phosphatase enzyme in skeletal muscle. Adipose tissue has a rather poor uptake of glucose; 0.4–2% of total tissue uptake is recovered in fat following an intravenous load of 25 g of glucose (spiked with 50 μCi of glucose- $\text{U-}^{14}\text{C}$) (Bjorntorp *et al.*, 1971). However, not all fat is the same (Harris and Leibel, 2008). Individuals who store excess fat subcutaneously may never experience diabetes as opposed to individuals who suffer excess visceral fat (e.g., omental, mesenteric, and renal) and ectopic accumulation of fat in, for example, liver and skeletal muscle (Samocha-Bonet *et al.*, 2012).

just under the skin. The latter means that glucose is measured in interstitial fluid, rather than blood, and some of the issues arising from measurements of glucose in interstitial fluid include the delay in glucose transfer from blood, the actual positioning of the sensor on the patient, and biofouling of the sensor. Recently, a lag between 5 and 6 min of radiolabeled glucose tracer appearance in interstitial fluid following an intravenous bolus administration was reported in healthy individuals in a controlled environment (Basu *et al.*, 2013), which may not necessarily present a confounding factor to continuous blood glucose monitoring depending, in part, on software algorithm used to calculate the glucose. The positioning of a glucose sensor, particularly if it is exposed to pressure during sleep, was found to result in aberrant glucose readings (Mensh *et al.*, 2013). The use of poly(ethylene glycol) polymer (Israelachvili, 1997; Sheth and Leckband, 1997) was successful in reducing the biofouling of the sensor by reducing protein adsorption and blood clot formation (van den Bosch *et al.*, 2013).

In contrast to above-mentioned medical devices that require hardware, software and power a polymer-based glucose-sensing and insulin-dispensing system has been developed. Nanoparticle-based three-dimensional scaffold responsive to hyperglycemia was designed to act as a self-sustained glucose-sensing and insulin-releasing system (Gu *et al.*, 2013b). Acid-degradable dextran was used as a matrix material for nanoparticles, which were then coated with polyelectrolyte chitosan (size: $\sim 335\text{--}345$ nm, average hydrodynamic radius peak at 340 nm, positively charged) or alginate (size: $\sim 285\text{--}300$ nm, average hydrodynamic radius peak at 293 nm, negatively charged). The particles were loaded with insulin, glucose oxidase, and catalase. Electrostatic interaction between chitosan (zeta potential $+10.6 \pm 1.9$ mV) and alginate (zeta potential -11.5 ± 1.7 mV) coated particles led to particle self-assembly into a network of nanoparticles consisting of agglomerates (opposite charge attraction) and pores (same charge repulsion). Glucose oxidase enzyme was incorporated to oxidize

glucose to gluconic acid, whereas catalase enzyme eliminated hydrogen peroxide from the glucose oxidation reaction and generated oxygen (needed for glucose oxidation). Gluconic acid from glucose oxidation served as a pH stimuli for the breakdown of dextran matrix and release of insulin, which in the presence of high glucose (400 mg dl^{-1}) showed a degradation within 8 h *in vitro*, whereas glucose levels of 100 mg dl^{-1} did not lead to detectable degradation of the system (Figure 6). The system was tested in type 1 diabetic mice, which retained insulin sensitivity 6 days post subcutaneous injection of the nanoparticles containing enzymes and the insulin compared to insulin only controls (Gu *et al.*, 2013b). Strategies such as the ones discussed earlier

could prove extremely useful as they would minimize/eliminate the need for frequent patient glucose measurement or insulin administration. Pending the stability of the polymer system, and fail-safes to guard against hypoglycemia, polymer-based systems such as the one developed by Gu *et al.* could offer an elegant way of long-term glucose management.

Lastly, isolation and transplantation of human islets of Langerhans have been actively pursued following the success of Edmonton protocol (Shapiro *et al.*, 2000). Paucity of islet tissue and loss of islets during the isolation and in the post-isolation period due to cell death remain some of the challenges of this procedure (Vantyghem *et al.*, 2011). Approaches aimed at alleviating

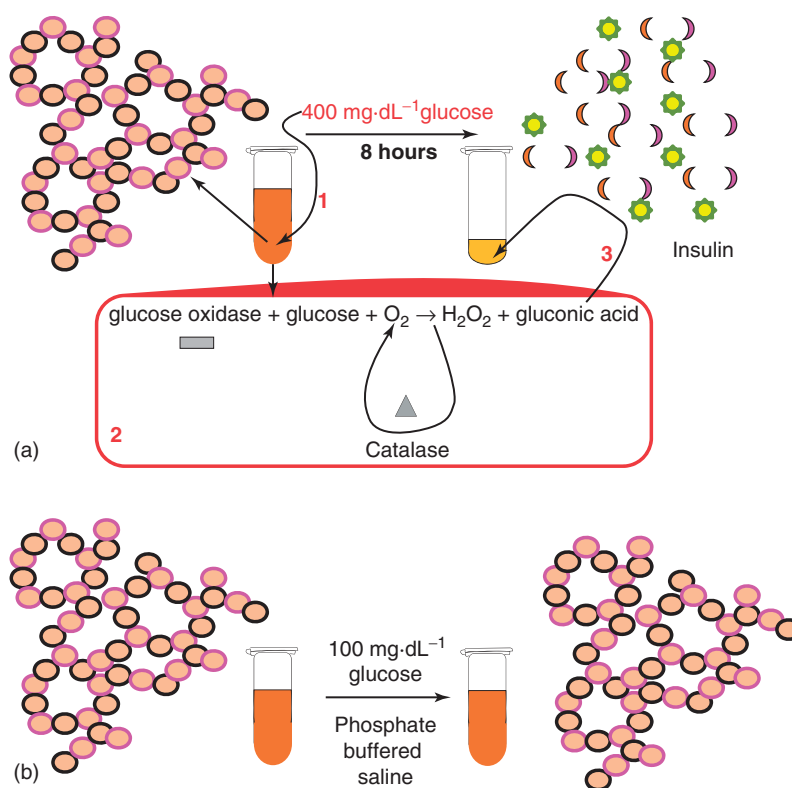


Figure 6. Hyperglycemia-sensitive and insulin-releasing nanoparticle-based nanonetwork. Incubation of nanoparticles made of acid-degradable dextran-matrix, coated with alginate (O) or chitosan (●), and loaded with insulin (★), glucose oxidase (▬), and catalase (▲) leads to insulin release under hyperglycemia conditions. Over a period of 8 h, nanonetwork exposed to 400 mg dl^{-1} glucose in eppendorf tubes resulted in dissociation of the nanonetwork with eventual total hydrolysis of acid-degradable dextran into native dextran, and release of insulin (a). The pH stimulus for degradation was provided by gluconic acid, which is obtained by glucose oxidation. Catalase was used to rid the reaction of hydrogen peroxide and to facilitate the glucose oxidation by recovering oxygen. Incubating the nanonetwork in phosphate-buffered saline or in 100 mg dl^{-1} glucose did not result in detectable loss of network's integrity (b). Illustration modified from (Gu *et al.*, 2013b).

some of these issues include the application of nanoscience and nanotechnology (Ghosh *et al.*, 2012; Kizilel *et al.*, 2010; Savic *et al.*, 2009; Zhi *et al.*, 2012). Zhi and colleagues have provided an *in vivo* proof of principle of nano-coated mouse islets to maintain normoglycemia in C57BL/6 mice with streptozotocin-induced diabetes (Zhi *et al.*, 2012). Islets isolated from Balb/c mice were individually coated with layers of charged polysaccharides. First, phosphorylcholine-modified chitosan (+) was adsorbed to islets, followed by deposition of alginate (−)—repeated several times—finally, the last deposited layer was that of phosphorylcholine-modified chondroitin-4-sulfate resulting in eight-layer coated islets. The islets ($N = 300$) were implanted under the kidney capsule of diabetic mice and mice followed up to 40 days after the procedure. Within 2–4 days, the implanted islets reduced the blood glucose levels, with majority (5/7) of the animals maintaining normoglycemia throughout the study, and reversing to hyperglycemia only after nephrectomy (>30 days). Two animals reverted to hyperglycemia without nephrectomy suggesting potential issues with encapsulation or loss thereof post implantation. Control mice with nonencapsulated islets reverted to hyperglycemia within 2 weeks of implantation suggesting rejection of Balb/c mouse islets by C57BL/6 recipient mice. Importantly, 4 weeks after implantation, C57BL/6 mice with nano-encapsulated Balb/c islets showed the same blood-glucose/time profile in response to 2 g kg^{-1} glucose challenge as did normal nondiabetic non-transplanted mice (Zhi *et al.*, 2012). These studies demonstrate the potential of nanocoating to preserve the function of islets and protect against immune reaction of the host.

Approaches to address the supply of islets include attempts to coax the islets to proliferate, which normally happens during pregnancy (Rieck and Kaestner, 2010). This has recently been achieved with some success by manipulating the expression of liver-derived betatrophin protein (Yi, Park, and Melton, 2013). Three perspectives describing the importance of these results are available (Crunkhorn, 2013; Kugelberg, 2013; Seymour and Serup, 2013). An alternative approach involves the use of stem cells (Dominguez-Bendala *et al.*, 2012; Efrat and Russ, 2012; Li, Gu, and Zhu, 2012). Stem cells are extensively covered within this book, and readers can be informed on stem cells applications in other

areas of medicine as well as drug discovery and toxicity testing and the effects of nanosystems on their physiology (see Chapter 17). The latter is particularly applicable to diabetes as one could envision the use of nanotechnological and regenerative medicine approaches in the development of stem cell-derived insulin-producing cells and their successful implementation in practice (Dominguez-Bendala *et al.*, 2012; Opara *et al.*, 2010). At the moment, islet transplantation procedures are not performed routinely and they remain restricted to clinical research centers and trials (NDIC, 2013). An enhanced, reproducible, and economically justifiable generation of islet cells from progenitors and/or stem cells, in particular beta cells, will likely lend these cells to applications in clinical treatment of diabetes.

6 CONCLUDING REMARKS AND FUTURE DIRECTIONS IN APPLICATION OF NANO IN DIABETES

During the writing of this review, it became apparent that advances in nanotechnology have not always translated into marketed products for the treatment and management of diabetes. The successful application of blood glucose sensors developed by utilizing the advances in nanotechnology will likely depend on their cost and ease of use by the patient. Genomics will continue to have an important influence, and may in fact quite rapidly produce novel causal links between genetics and diabetes, which will help drive the development of better screening and diagnostic tools. Outside of insulin variations and delivery systems, nanomedicines are few. This may change with the current trend of repurposing of drugs, which is endorsed by National Institutes of Health (NIH, 2013b), requiring the collaboration of systems biology and pharmacology experts along with other health care professionals (Chandra and Padiadpu, 2013; Dudley, Deshpande, and Butte, 2011; Gero *et al.*, 2013; Sirota *et al.*, 2011). The promise of an artificial pancreas has come through in a form of battery-powered medical devices that sense blood glucose and insulin pumps that dispense insulin. The use of polymers in manufacturing glucose-responsive, insulin-dispensing polymer networks—which do not have or need electrical wiring or power—may alleviate the present

energy dependence of current devices, and potentially offer a more comfortable therapeutic approach for patients (Gu *et al.*, 2013b).

The most important advance seems likely to come from the early detection of the disease. The promise of nanotechnology anticipated from the results emerging from the ongoing physical and health sciences researches is the development of simple and inexpensive monitoring and treatment devices and approaches that will dramatically impact the detection and treatment of people with prospective and frank diabetes.

Biocompatibility and long-term safety of nanomaterials and nanotechnologies aimed at human use will have to be satisfied. Expert reviews on the topics of chronic nanotoxicity, immunotoxicology, and methods of examining the toxicity can be found elsewhere in this book (*see* 3, 1, 5). Regulatory agencies such as FDA will play a major role in getting the products to the market, which may be challenging in the beginning but we expect that it would become less so, as more and more technologies reach the market, and FDAs' taskforce manages the regulation of such devices and technologies (FDA, 2013c).

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Inhalation Pathway as a Promising Portal of Entry: What Has to Be Considered in Designing New Nanomaterials for Biomedical Application?

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1 INTRODUCTION

Animals as well as humans are constantly exposed to particles of different sources in the environment. Humans inhale millions of different-sized particles with every breath [reviewed in (Gehr *et al.*, 2010)]. The possible portals where particles may enter the human body are the lungs via inhalation, the gastrointestinal tract via digestion, the skin, and blood vessels via intravenous injection (Oberdorster, Oberdorster, and Oberdorster, 2005). Owing to its vast epithelial surface area (Gehr, Bachofen, and Weibel, 1978), the lung is considered by far to be the most important portal of entry for particles into the human body. This is a disadvantage in the case of particles causing diseases or adverse effects such as ambient and/or combustion-derived particles as it has been recognized as an important cause of cardiovascular morbidity and mortality in areas with air pollution (Mills *et al.*, 2009; Wichmann *et al.*, 2000; Oberdorster, 2001). However, concurrently, the

lung characteristic is also considered as a promising pathway for biomedical applications using the inhalation pathway to deliver new nanomaterials. Nanosized carriers [e.g., mainly nanoparticles with all three dimensions below 100 nm (ISO/TS, 2008)] have been proposed as promising novel diagnostic, therapeutic, and vaccination approaches for a variety of human diseases (Zrazhevskiy, Sena, and Gao, 2010; Nembrini *et al.*, 2011; Foged *et al.*, 2005).

Already for more than 50 years, the pulmonary uptake route has been used as a method for administering medicinal therapies. Inhaled drugs are already applied to treat airway diseases, are in development, or have been considered for the treatment of several other lung diseases. In 2004, there were more than 25 drugs licensed for use in treating pulmonary diseases, such as asthma, emphysema and chronic inflammation, chronic obstructive pulmonary disease (COPD), and lung cancer (Patton, Fishburn, and Weers, 2004).

About 10 years ago, there were no inhalable drugs for systemic diseases on the market but at that time it was assumed that this was about to change. Several clinical trials started with inhaled insulin, and a variety of other different sized molecules was under investigation as inhaled formulations for systemic applications (Patton, Fishburn, and Weers, 2004).

However, in the recent medical history, this method of delivery has fallen out of use in favor of methods that generally provide a more direct entry into the system circulation, such as injection. With the development of nanomaterials (i.e., a material with one, two, or three external dimensions in the nanoscale (<100 nm) (ISO/TS, 2008)) for drug delivery, medical imaging, and diagnostic purposes, the inhalation pathway is becoming more important again. The lung is being reconsidered as a possible noninvasive method for the delivery of drugs because of its large surface area and generally rapid uptake process into the systemic circulation without the problem of the first-pass effect^a (Jud *et al.*, 2013). In addition, the small size of nanomaterials may allow for greater permeability of nonsoluble compound (drugs and proteins) across the air–blood tissue barrier (Kreyling *et al.*, 2012). Pulmonary delivery provides much faster absorption of small molecules, with much less metabolism when compared to the gastrointestinal tract (Patton and Byron, 2007). Overall, the benefits of systemic drug delivery through the lungs typically include a rapid onset of action and an increased bioavailability over oral formulations (Malik *et al.*, 2007; Shoyele and Slowey, 2006; Bailey and Berkland, 2009).

The significant potential of the inhalation pathway as a delivery route has now been realized. Thus, the inhalation pathway is a very promising route for the development of new biomedical applications based on specifically designed nanomaterials used as drug delivery vehicles for diagnostic and therapeutic treatments and may revolutionize medicine. The knowledge about the anatomical and cellular structures of the human respiratory airways as well as the nanomaterial–lung cell interactions is, however, mandatory in order to understand the engineering of specific nanomaterials/nanocarriers. In addition, it is also of high importance to be aware of and investigate the potential off-target effects associated with each new nanomaterial before the application can be introduced to the market.

2 ANATOMY OF THE HUMAN RESPIRATORY SYSTEM

This part highlights certain aspects of lung structures, which are most relevant regarding the function as a portal of entry for nanomaterials. For a more comprehensive and detailed description of the respiratory tract related to morphology, cytology, histology function, and structure, we recommend the book chapter of Ochs and Weibel in *Fishman's Pulmonary Diseases and Disorders* (Ochs and Weibel, 2008) and the report of the International Commission on Radiological Protection (ICRP, 1994)

2.1 General Anatomy of the Respiratory Tract

The respiratory tract offers a huge internal surface area of about 150 m^2 (i.e., alveoli and airways) (Gehr, Bachofen, and Weibel, 1978) that is optimized and essential for a sufficient gas exchange in the human body. The main function is to allow oxygen to move from the air into the venous blood and for carbon dioxide to move out. Anatomical regions of the respiratory tract can be subdivided into four parts (Figure 1):

1. the extra thoracic region comprising the anterior nose and the posterior nasal passages, larynx, pharynx, and mouth;
2. the bronchial region consisting of the trachea and bronchi from which inhaled particles are cleared by mucociliary action;
3. the bronchiolar region consisting of bronchioles and terminal bronchioles; and
4. the alveolar–interstitial region consisting of respiratory bronchioles (bronchioles with some alveoli apposed), the alveolar ducts and sacs with their alveoli, and the interstitial connective tissue.

All four regions contain lymphatic tissue or its components (ICRP, 1994).

After inhalation, the airflow follows the airways in a series of regularly dichotomously branching tubes, which are getting shorter and thinner toward the peripheral regions. The model of regular dichotomous (Figure 2) branching of human airways from the trachea (generation $z=0$) to the alveolar ducts and sacs (on the average generation 23) can be explained as follows: the first 14 generations are

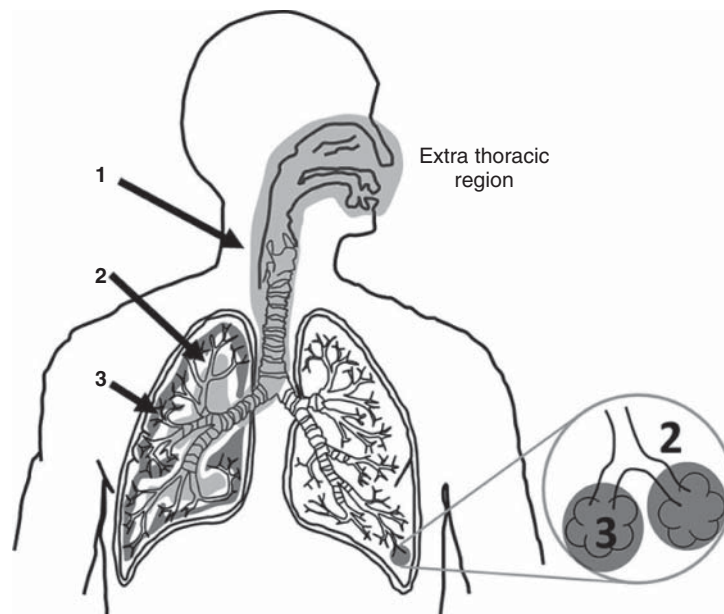


Figure 1. The human respiratory tract can be subdivided into four main structurally and functionally distinct areas. The air enters the thoracic region via the extra thoracic region and continues to the tracheobronchiolar region (1, light gray). From there, the air is conducted to the proximal part of the alveolar–interstitial region (2, white) before it reaches the distal part of the alveolar–interstitial region (3, dark gray). [C. Jud *et al.* (2013). Reproduced by permission of Swiss Medical Publishers Ltd.]

pure conducting airways and are lined by a pseudostratified epithelium. In the last nine ($Z' = 0-8$), generations alveoli are opposed and gas exchange takes place. In generation 15 ($Z' = 0$), first alveoli appear at the airway wall and therefore this generation represents the change from the conducting to the respiratory region (Ochs and Weibel, 2008; Weibel, 1963).

2.2 Airway Epithelium

The airway epithelium lines the entire airways without any interruption down to the generation 18. Although it originates from only one anlage, the airway epithelium changes its characteristics on the way down concerning architecture and cell types. Starting at the level of trachea/bronchi, the epithelium is pseudostratified, and at the level of smaller bronchioles, it is of cuboidal appearance (Figure 3). Moving down toward the alveoli (alveolar ducts), the epithelium becomes extremely thin (Ochs and Weibel, 2008; Weibel, 1963). Generations 19–23 (alveolar ducts and alveolar sacs) are

lined by squamous cells, the alveolar type I epithelial cells that share a basement membrane with the pulmonary capillaries. The alveoli also contain alveolar type II epithelial cells, which secrete lung surfactant (surface active agent) to prevent alveolar collapse (Daniels and Orgeig, 2003). The structural barrier between air and blood is reduced to an approximately 2- μm -thick tissue layer in the alveoli (Gehr, Bachofen, and Weibel, 1978). More than 40 different cell types, among others, different types of epithelial cells, endothelial cells, fibroblasts, nerve cells, lymphoid cells, gland cells, dendritic cells, and macrophages, add to the complexity of the epithelium in the lung (McClellan, 2000; Ochs and Weibel, 2008).

There are approximately 300 million alveoli in the lungs, with a combined surface area that is about 150 m^2 and with an alveolar epithelium as thin as 0.1 μm (Gehr, Bachofen, and Weibel, 1978; Brain, 2007; Weibel, 1963). This large surface area, combined with an extremely thin barrier between the pulmonary lumen and the capillaries, creates conditions that are well suited for efficient mass and gas transfer (Brain, 2007).

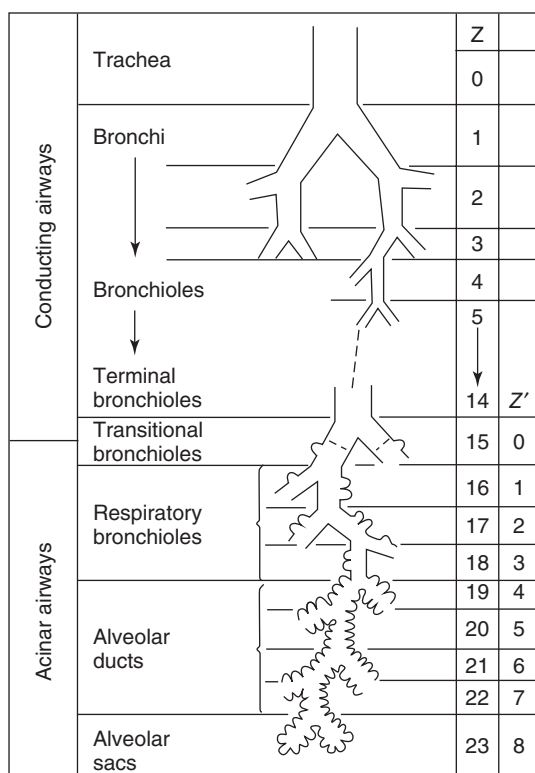


Figure 2. Model of airway branching in human lung by regularized dichotomy from trachea to alveolar ducts and sacs. [E. Weibel (2009) Reproduced by permission of Swiss Medical Publishers Ltd.]

3 NANOMATERIAL–LUNG INTERACTIONS

Knowledge of general particle–lung interactions is necessary for the design of new nanomaterials for medical applications especially considering the target drug's place of action. Thus, a short overview is given about particle deposition, clearance mechanism in the lung, and particle translocation into the blood circulation. Furthermore, the natural barrier system will be discussed. This prevents (adverse) foreign material from entering the body but has to be overcome in the case of nanomaterial design. As most of the studies about interaction of inhaled (nano)materials and the lung have been done with particles, this chapter summarizes findings about particle–lung interactions.

3.1 Particle Deposition

In daily life, the lungs are in direct and constant contact with the ambient air. Particles from very different sources with different size, shape, and material at different concentrations are inhaled with every breath (Jud *et al.*, 2013). According to the particle size, it can be predicted in which compartment they will be predominantly deposited in the lung (Patton and Byron, 2007; Heyder *et al.*, 1986). The fractional deposition of inhaled particles in the nasopharyngeal, tracheobronchiolar, and alveolar region of the respiratory tract under conditions of nose breathing during rest is described in Patton and Byron (2007).

Particles with an aerodynamic diameter $>10\mu\text{m}$ cannot enter the lung and are filtered out in the nasopharyngeal region of the respiratory tract. Particles that are smaller than $10\mu\text{m}$ and enter the lung can be deposited by inertial impaction, sedimentation, or diffusion.

3.1.1 Impaction

Inertia is the inherent property of a moving mass to resist acceleration. It may cause particles to move in their original directions and not follow airflow streamlines, such that they deposit on airway walls by impaction. The inertia of a particle depends not only on the particle density and size but also on the airflow velocity. Impaction occurs in regions where flow velocity is high and rapid changes in airflow direction occur. Hence, deposition by impaction is found in the extrathoracic and large conducting airways (Schulz, Brand, and Heyder, 2000).

3.1.2 Sedimentation

Particles are continually exposed to gravity and undergo gravitational settling. In contrast with diffusional transport, particle displacement by gravitational settling becomes significant for particles with an aerodynamic diameter larger than $0.5\mu\text{m}$. The distance a particle settles within a given time increases with its mass (i.e., with its density and diameter). The longer a particle remains in the respiratory system the larger is the settling distance a particle will cover and hence the probability that the particle will touch the airspace wall, as it is the case in the small conducting airways and gas-exchange area (Schulz, Brand, and Heyder, 2000).

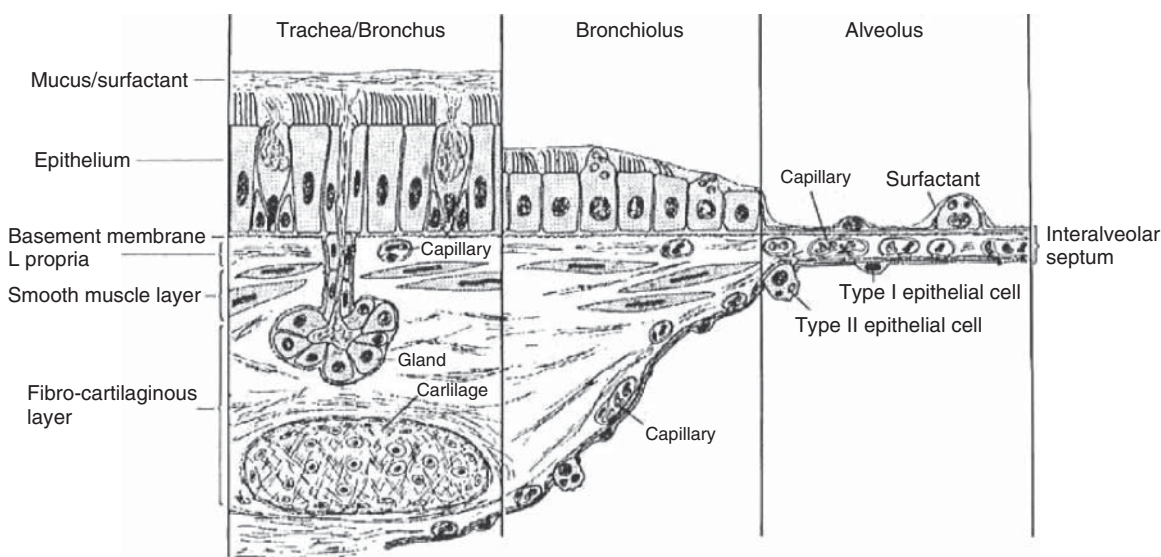


Figure 3. Airway wall structure at the three levels—trachea/bronchus, bronchiolus, and alveolus. [Ochs, M. and Weibel, E.R., Functional design of the human lung for gas exchange, 2008, McGraw-Hill. Reproduced with permission of the McGraw-Hill companies.]

3.1.3 Diffusion

For particles with an aerodynamic diameter $<0.5 \mu\text{m}$, particle displacement is governed mainly by diffusional transport. Collisions between gas molecules and particles cause numerous very small random displacements of particles. The distance a particle will travel by diffusion increases with time and with decreasing particle diameter. Hence, the probability for particles to hit airspace surface by diffusional transport is larger the smaller the particles are and the longer they remain in the respiratory tract. This will occur mainly in the lung periphery (Schulz, Brand, and Heyder, 2000).

Thus, a consequence for nanomaterials that are designed for the inhalation route is that the materials have to be adjusted in size. A particle deposition subject to inertial impaction in the nasopharyngeal region of the respiratory tract, or sedimentation in the bronchial region, delivered drug may be expected to have little systemic therapeutic effect (Brain, 2007; Edwards and Dunbar, 2002; Sung, Pulliam, and Edwards, 2007). At the other extreme, it is known that a large amount of inhaled nanomaterials are exhaled without deposition and another fraction is deposited on the internal walls of the lung by diffusion (Schulz *et al.*, 2005; Limbach *et al.*, 2007). It should, however, be noted that evidence has been published that nanoparticles can also deposit

in the olfactory epithelium and directly translocate to the brain (Oberdorster, Elder, and Rinderknecht, 2009). Taken all this information together, for most of the biomedical applications using nanomaterials, a size range of 50–150 nm for which diffusion is the dominant process is assumed to be optimal.

3.2 Displacement of Deposited Particles

While being deposited, particles first come into contact with the lung surfactant, which is located at the air–liquid interface of a liquid lining layer covering the cells at the luminal side of the wall. Particles are then displaced into the aqueous lining layer (airway) or hypophase (alveoli) by wetting forces where they may interact with cells, that is, epithelial cells or cells of the defense system (Schurch *et al.*, 1990; Gehr *et al.*, 1990; Gil *et al.*, 1979). They may even be translocated into the capillaries and reach extra pulmonary sites by the bloodstream (Mühlfeld, Gehr, and Rothen-Rutishauser, 2008; Elder *et al.*, 2006; Nemmar *et al.*, 2002).

In the case of ambient particles or particles inhaled occupationally, a translocation can cause harm. Therefore, the respiratory tract has clearance and several defense mechanisms against the entry of xenobiotics, which may have an adverse effect on the human body. However, in the case of inhaled

nanomedicine, these defense mechanisms have to be overcome.

3.3 Clearance of Deposited Particles in the Lung

The clearance of deposited particles in the respiratory tract is dependent on the particle material and as a result of the size, dependent on the site where the particles have been deposited. Clearance includes basically two processes: (i) a physical clearance of particles and (ii) chemical clearance processes (Oberdorster, Oberdorster, and Oberdorster, 2005; Kreyling and Scheuch, 2000),

1. Physical clearance processes are as follows:
 - a) mucociliary movement (nasal and tracheobronchial)
 - b) macrophage phagocytosis (tracheobronchial and alveolar)
 - c) epithelial endocytosis (nasal, tracheobronchial, and alveolar)
 - d) interstitial translocation (tracheobronchial and alveolar)
 - e) lymphatic drainage (tracheobronchial)
 - f) blood circulation (tracheobronchial and alveolar)
 - g) sensory neurons (nasal and tracheobronchial)
2. Chemical clearance processes (nasal, tracheobronchial, and alveolar) are as follows:
 - a) dissolution
 - b) leaching (loss of elements from the particle matrix)
 - c) protein binding.

The above-mentioned lists clearly indicated that the clearance depends on the lung compartment where particles have been deposited. Both long-term and short-term particle elimination from conducting airways and the lung periphery have to be considered. In summary, the particles can be eliminated from airways by mucociliary transport (fast process) and by penetration into the epithelia of the bronchial conducting airways and the lung periphery (slow process). For both processes, chemical clearance, for example, dissolution in mucus or inside of cells, may interfere with physical clearance.

3.3.1 Clearance from Conducting Airways

The airway epithelial goblet cells and submucosal glands secrete mucus, forming a two-layer mucus blanket over the ciliated epithelium, that is, a low-viscosity sol layer covered by a high-viscosity gel layer. Insoluble particles are trapped in the gel layer and are moved toward the pharynx (and ultimately to the gastrointestinal tract) by the upward movement of mucus generated via metachronous beating of the cilia. This mechanism is called *mucociliary clearance* (Kilburn, 1968).

In the normal lung, the rate of mucus activity varies depending on the airway area and is determined by the number of ciliated cells and their beat frequency. Mucus transport velocities are between 10 and 20 mm min⁻¹ in the trachea (Morgan *et al.*, 2004). It was extrapolated that deposited particles are cleared within about 24 h. For normal mucociliary clearance to occur, the airway epithelial cells, the ciliary structures, and activity must remain intact. Furthermore, the depth and chemical composition of the sol layer should be optimal and finally the rheology of the mucus must also remain within the physiological range. Mucociliary clearance is impaired in lung diseases such as immotile cilia syndrome, bronchiectasis, cystic fibrosis (CF), and asthma (Houtmeyers *et al.*, 1999). A recent study has shown that nanoparticle mobility in the mucus is dependent on adhesive or inert properties of the material (Kirch *et al.*, 2012).

3.3.2 Clearance from the Lung Periphery

The clearance kinematics in the lung periphery is much slower because of the absence of mucociliary action. In the lung periphery, the particles can be eliminated by (i) phagocytosis and subsequent transport by macrophages, (ii) dendritic cells with a subsequent transport to the draining lymph nodes, and (iii) translocation via the alveolar epithelium into the bloodstream. All these mechanisms by which the particles are eliminated from the respiratory tract have to be considered for the design of new inhaled drugs (Moller *et al.*, 2010).

3.4 Cellular Components of the Pulmonary Clearance System

The respiratory tract contains a series of structural and functional barriers and the fate of the deposited

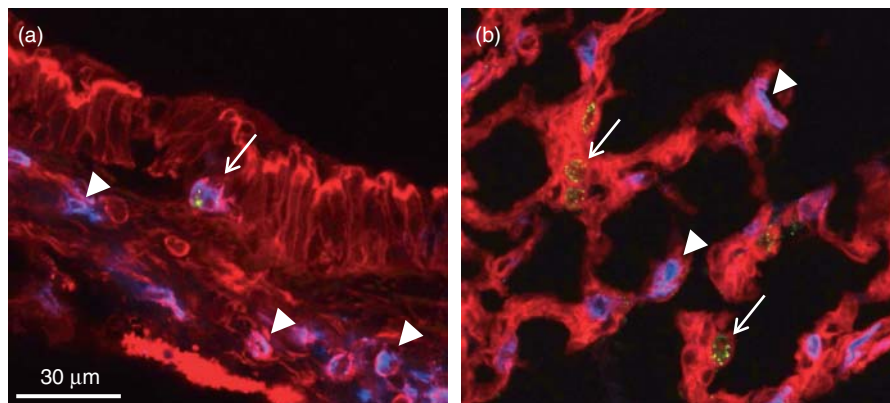


Figure 4. Different respiratory tract compartments of a mouse visualized by laser scanning microscopy. Trachea (a) and lung parenchyma (b) were collected 24 h after intranasal administration of 24 nm polystyrene nanoparticles (yellow). F-actin in the cells is shown in red, dendritic cells in blue (major histocompatibility complex II label, arrowheads), and instilled nanoparticles in yellow. Note the pseudostratified epithelium in the trachea (a) compared to the squamous alveolar type I and II cells in the parenchyma (b). The nanoparticles were detected within dendritic cells (a, arrow) and in macrophages (b, arrows).

nanomaterials depends not only on their physicochemical characteristics but also on the exposed cell type, that is, phagocytes (macrophages), antigen-presenting cells, epithelial cells, or endothelial cells (Von Garnier, Rothen-Rutishauser, and Blank, 2013).

The conductive as well as the gas-exchange regions are lined by a continuous epithelium (Figure 4). The epithelial cells are endowed with tight junctions (TJs) and adherens junctions (AJs) and desmosomes between the cells. The TJs are important for the epithelial integrity and prevent molecules and particles from easily crossing the epithelial barrier (Schneeberger and Lynch, 1984; Godfrey, 1997). In addition to the mechanical properties, there are liquid layers covering the surface that contains protective proteins (e.g., surfactant proteins A and D and immunoglobulins). As professional phagocytic cells, airway and alveolar macrophages belong to the first line of the cellular defense against inhaled and deposited antigens. The airway and alveolar macrophages are found within the mucus layer of the airways and the aqueous hypophase of the alveoli, respectively (Lehnert, 1992; Brain, 1988). Furthermore, a network of dendritic cells inside and underneath the epithelium belongs to the cellular defense system. They are professional antigen-presenting cells and are found to a larger extent in the proximal airways and are also present in the gas-exchange region, however, to a lesser extent (Holt and Schon-Hegrad, 1987;

McWilliam, Holt, and Gehr, 2000). As the respiratory tract contains millions of immune cells, it represents an ideal target organ for immune modulation by specifically designed nanomaterials (Von Garnier, Rothen-Rutishauser, and Blank, 2013; Stano *et al.*, 2012). Besides macrophages and dendritic cells, there are other immune cells in the airways, which play a less important role regarding particle uptake but are crucial for a proper immune response and interaction with other cells. For example, natural killer cells are important for the fighting of tumor cells and viral and bacterial infections (Vivier *et al.*, 2011) and cross talk with dendritic cells (Walzer *et al.*, 2005). In addition, T cells, one of the main cell types of the adaptive immune response, also communicate with dendritic cells and may be affected by medical nanoparticles (Blank *et al.*, 2011).

3.5 Cellular Uptake and Translocation of Particles

Despite these barriers, there are possibilities for particles to penetrate into the human body. Particles probably might cross the epithelial layer in a paracellular manner, for example, via transcytosis and other mechanisms [for details, see (Oberdorster, Oberdorster, and Oberdorster, 2005; Mühlfeld, Gehr, and Rothen-Rutishauser, 2008; Rothen-Rutishauser *et al.*, 2007)] and via the cells of the immune system, for example, by slender transepithelial cytoplasmic processes of macrophages and dendritic cells

(Blank *et al.*, 2010). The translocation through the airway epithelium is dependent on the particle's characteristics. These characteristics have to be considered for the design of new medication, for example, the knowledge that lipophilic molecules easily pass through the airway epithelium via passive transport. However, hydrophilic molecules cross via extracellular pathways and exocytosis (Summers, 1991). Particles are absorbed from the submucosal region into the systemic circulation, bronchial circulation, and/or lymphatic system.

3.6 Inhalation and Dosing of Drugs

The challenge for new biomedical applications is to meet the commercial requirements for a stable drug formulation in (ideally) an inexpensive, miniature device with the means of encouraging the user to inhale in a controlled way from the mouthpiece.

Aerosol clouds with optimal properties should contain nanomaterials that are neither too small (risk of exhalation of the material) nor too large (these deposit primarily in the upper airways, mouth, and throat) (Byron, 1986), which seems to be in the size range 50–150 nm. Currently, there are three types of inhalers on the market: nebulizers, dry powder inhalers, and metered-dose inhalers. Nebulizers are the oldest technology for delivering drugs to the lungs. A suspension or solution is aerosolized by a compression jet and consequently inhaled by the patient. Dry powder inhalers deliver a dry powder drug through induced aerosolization and are breath-actuated devices. Therefore, the actual dose delivered to the lung is dependent on the inspiration flow rate and a proper replication is the weak point. If patients inhale too forcefully from a dry powder inhaler, particle deposition in the upper airways, mouth, and throat is dominant and lung deposition falls. Metered-dose inhaler contains a drug suspension/solution and releases a metered volume of drug through a valve system. This inhaler type requires coordination between actuation and inhalation to ensure an optimal deposition in the lungs. Forceful inhalation from a pressurized metered-dose inhaler can actually increase lung penetration of an aerosol that would normally deposit primarily in the back of the throat (Bailey and Berkland, 2009; Patton and Byron, 2007). Thus, detailed instructions and training for the use of the devices are crucial for a successful and effective treatment.

3.7 Perfusion of the Lung and Distribution of Particles in the Body

As the lungs receive the entire cardiac output, they represent the most richly perfused organ in the body. However, only the alveolar region is supplied by the pulmonary circulation and the blood flow to the larger airways (trachea and bronchi) is via the systemic circulation (Nunn, 1993). Via the bronchial veins and the right atrium, the endobronchial circulation is recirculated to the peripheral airways. Theoretically, inhaled drugs that are absorbed into the circulation from the tracheobronchial regions can be redistributed downstream and peripherally absorbed drugs can reach otherwise poorly accessible areas of the lung (Deffebach *et al.*, 1987).

4 BIOAVAILABILITY

Besides the deposition site of the nanomaterials in the lung, which is mainly influenced by the size of the delivery package and the breathing pattern of the person, as well as the targeted cell(s), it is crucial whether the nanomaterial is bioavailable or not. Bioavailability is usually defined as the fraction/percentage of an administered drug or other substance that becomes available to the target tissue/blood after administration. Regarding bioavailability and biomedical applications, we have to distinguish between the different goals of the application:

- (A) **Local treatments with target in the lungs** Nanomaterial can target the lung itself and the active component needs to be bioavailable in the specific region of the lung.
- (B) **Systemic treatments** Nanomaterials are only applied via the lungs and should act systemically, which means that the active component needs to pass the air–blood tissue barrier and reach the blood or the lymph circulation.
- (C) **Specific target not in the lungs** Nanomaterials should reach a specific target (e.g., cancer cells) somewhere in the body. Nanomaterials are applied via the lung and need to be transported to the target without developing any (side) effects during transport.

Looking for optimal characteristics of nanomaterial for biomedical application, those different goals

need to be kept in mind. Thus, for targeting the lung itself (A), the nanomaterial needs to reach the compartment of the lung in which the treatment should be active and where the drug should be bioavailable to develop the desired effect. However, to achieve a systemic effect (B), the drug needs to reach the blood circulation, which requires passing the air–blood tissue barrier. The third situation (C) where the specific target is anywhere in the body, the nanomaterial needs to be designed to reach the blood circulation without releasing the drug. After achieving at the target (cells and organ), the drug has to be released from the nanocarrier and become active.

In general, the bioavailability of small molecules applied via inhalation is higher than through the gastrointestinal tract because the molecules are absorbed faster. In addition, the molecules are less altered by enzymatic activity of the first-pass metabolism (Patton and Byron, 2007). In addition, the absorption by the systemic circulation after deposition in the lung is the fastest route of any delivery route other than the intravenous one (Patton, Fishburn, and Weers, 2004).

Various characteristics of the nanomaterials such as size, hydrophobicity, solubility, and enzymatic hydrolysis define to what extent the material will be bioavailable.

4.1 Size

Small molecules (in the size range of only a few kilodaltons) can reach pulmonary bioavailability of over 90% (Tronde *et al.*, 2003) because of their rapid absorption and the—compared to the oral-gastrointestinal route—low concentrations of metabolic enzymes in the lung that keep the metabolism of the molecules low. The absorption half-life² can reach values lower than 1 min but can also be more than 30 min (Brown and Schanker, 1983). For small molecules, the size alone does not predict the bioavailability, also the hydrophobicity plays a crucial role (for details, see Section 4.2). This is different for macromolecules where the absorption is primarily dictated by their size. The larger the size of the molecule, the slower is the absorption. Typical macromolecules (such as insulin, growth hormone, and several cytokines) reach their peak in the blood circulation 30–90 min after the aerosol inhalation (Patton and Byron, 2007). Even though the absorption of macromolecules in the lungs

is slower than for small molecules, bioavailability for macromolecules provided by the lungs is higher than in any other noninvasive delivery route (Dershwitz *et al.*, 2000; Patton and Byron, 2007). Furthermore, it has to be considered that proteins with a molecular weight higher than 150 kDa have difficulties to reach the blood circulation as the transport of proteins through the air–blood barrier seems to decrease with increasing molecular weight (Bailey and Berkland, 2009).

Macromolecules of several 100 kDa can be seen as very small particles in the range 5–15 nm in diameter for globular structure.

4.2 Hydrophobicity

With increasing hydrophobicity, the solubility decreases and the absorption of nanomaterials in the lungs gets slower. Insoluble molecules can persist in the lungs for weeks. The highest absorption kinetics is found for small molecules, which are mildly hydrophobic. Small hydrophilic molecules are absorbed less rapidly than hydrophobic ones but still faster than via the oral route (Patton, Fishburn, and Weers, 2004). For macromolecules, the hydrophobicity is less important and the size is what primarily defines the absorption. Adding an aliphatic tail group to the nanomaterial increases the hydrophobicity, which, in turn, decreases the aqueous solubility (Bailey and Berkland, 2009).

4.3 Solubility

The solubility of nanomaterials needs to be balanced as a solubility is required that is neither too high nor too low. If a nanomaterial is too soluble, the nanomaterial is absorbed very quickly and the systemic duration is very short. This means that the nanomaterial does neither have a local effect in the lungs nor is the systemic efficiency high. On the other hand, insoluble nanomaterial is either removed from the lungs within 24 h by the muciliary clearance if it was deposited in the conducting airways or engulfed by macrophages if it was in the alveolar region (Patton and Byron, 2007). In both cases, the nanomaterial is not bioavailable any longer and thus is without any effect on the human body.

In order to achieve the solubility needed for a certain application, the solubility of nanomaterial can be decreased by either adding a hydrophobic

molecule or conjugation with a water-soluble inert ligand [e.g., polyethylene glycol (PEG) (Leach *et al.*, 2004), Fc region of IgG1 antibody (Bitonti *et al.*, 2004, Low *et al.*, 2005)]. Insulin, for example, can be formulated either as a liquid or in highly water-soluble aerosol particles that dissolve rapidly in the lung (Patton and Byron, 2007).

4.4 Enzymatic Hydrolysis

Using the oral route of delivery, the gastrointestinal mucosa and the condition in the gastrointestinal tract have to be considered. Even though, a lot of the concepts and findings for the gastrointestinal mucosa can be transferred to the respiratory mucosa, there are also important differences. One important difference is the activity of the hydrolytic enzymes with regard to biomedical applications and delivery of drugs. While in the gastrointestinal tract, the enzymatic activity is high and can attack all kind of molecules, whereas in the lungs, the metabolism is reduced. For small molecules, the lung metabolism is minimal and can be neglected in most cases, but macromolecules and also small peptides may be subject to enzymatic degradation, also in the lung. Even though, the environment is still much more hospitable to proteins and nucleic acids than in the gastrointestinal tract (Bailey and Berkland, 2009). To overcome the enzymatic activity that can attack natural peptides, the peptides can be blocked to reduce the sensitivity to peptidases (Patton, Nagarajan, and Clark, 1998). Gold nanoparticles can be functionalized with quaternary ammonium groups that bind to negatively charged DNA or RNA and protect the nucleic acid from enzymatic degradation (Han *et al.*, 2007). For bigger peptides, or proteins with tertiary and quaternary structure, the peptidase hydrolysis is inhibited or even eliminated, which makes natural protein highly bioavailable (Patton, Nagarajan, and Clark, 1998).

Depending on the aim, the characteristics described hereby have to be considered to produce tailor-made nanomaterials.

5 APPLICATION FIELDS: CASE STUDIES OF CURRENT APPLICATIONS AND FUTURE POTENTIAL

The use of the inhalation pathway for medical treatments has a long history. Already before Christ,

the Egyptians used “vapors” in medical treatment; in the seventeenth century, anno Domini an inhalation treatment was used for Tuberculosis; and in the nineteenth century, Asthma was treated with inhalation and the first portable nebulizer was developed (Patton and Byron, 2007). Later, in the twentieth and twenty-first centuries, the research on inhalation treatments and development of inhalation devices literally exploded, and nowadays, there are various device types and numerous medical applications on the market. However, most of them are for the local treatment of the lungs, some for the systemic treatment, and only very few for the treatment of specific local targets in other organs. Especially for the last and also the second last type of medical applications, new engineered nanomaterials are considered as optimal for the development of new drugs or drug carriers as the physicochemical properties can be tuned for the desired compartment and bioavailability.

The following five case studies present current applications for the three different types (local lung treatment, systemic effect and local target not in the lung) as well as an outlook of the future potential.

5.1 Local Treatments with Target in the Lung

5.1.1 Asthma Treatment with Corticosteroids

The following information is summarized from Dahl (2006) and Derendorf *et al.* (2006). Corticosteroids are an effective treatment of Asthma and can reduce airway inflammation and hyperresponsiveness, improve the lung function, decrease the symptom severity, and prevent/reduce the occurrence of acute exacerbations. The use of inhaled corticosteroids is the treatment of choice for chronic asthma. Corticosteroids act through an interference with multiple inflammation pathways. They diffuse across the cell membrane of the target cell and bind to the glucocorticoid receptor in the cytoplasm. The binding activates the glucocorticoid–receptor–corticosteroid complex that subsequently translocates into the nucleus. There the complex binds to specific DNA sequences that alter the gene transcription and protein synthesis associated with inflammation.

Despite the delivery of glucocorticoids via inhalation directly to the lung, where it acts locally, there can be local and systemic side effects. The

efficacy and safety of the drug depends on the material characteristics. Inhaled corticosteroids with a high pulmonary availability usually also have a high potential for unwanted (systemic) side effects because most of the drug available in the lungs can reach the circulation. Higher receptor-binding affinities and longer half-lives also correlate positively with increased potential for systemic side effects. The particle size needs to be optimized to reduce the local side effects. The goal is to have as much drug deposited in the lungs as possible while minimizing the deposition in the oral cavity. The lipophilicity of the inhaled corticosteroids facilitates the passage of the drug and positively not only correlates with the pulmonary retention time and the volume distribution of the drug but also alters the distribution after systemic absorption. Lipid conjugation provides a slow-release reservoir of the inhaled drug in the target tissue and prolongs lung residence time. Inhaled corticosteroids can bind to intracellular proteins or to proteins present in the fluids of the circulation. Most common is the binding to albumin, which occurs rapidly and is reversible. The degree of protein binding helps to control the systemic concentration of unbound corticosteroids and therefore limits the systemic side effects. Last but not least, the clearance of the drug also needs to be considered. Inhaled corticosteroids are rapidly cleared primarily by the liver and also by other organs.

For a safe, but efficient and user-friendly treatment with inhaled corticosteroids, the delicate balance between efficacy and safety is important. Thus, the use of nanomaterials with distinct characteristics for the development of new—at the same time—safer and more efficient applications could be very valuable.

5.1.2 Antibiotic Treatment of *Pseudomonas* in Cystic Fibrosis Patients

The following information is summarized from (Hewer, 2012) and (Pilcer *et al.*, 2013). CF is an inheritable (autosomal recessive) disease that primarily affects the lungs, pancreas, liver, and intestines. The lungs show airway inflammation and infections starting at an early age. Especially, bacterial infections are a problem and need to be treated with antibiotics. One of the most common bacterial infections in CF patients is *Pseudomonas aeruginosa*. While early *Pseudomonas* infection

usually is treated aggressively to try to eradicate it and prevent or at least delay the acquisition of a chronic infection, the chronic infection cannot be eradicated and therapy aims to suppress and control the infection. Three delivery routes (oral, intravenous, and inhaled) can be used for the antibiotic therapy from which the inhaled route has principle advantages: increased antibiotic concentration at the infection site, enhanced bacterial killing, and reduced systemic toxicity. Colistin and Tobramycin are the two most commonly used inhaled antibiotics and have already been used for years. However, recently new formulations of those antibiotics and especially new delivery devices have been developed. The new Tobramycin inhalation powder has to be administered twice daily and does not need power. A capsule is inserted and the Podhaler is activated manually. The patient inhales, holds the breath, and repeats the whole cycle for a total of four capsules. The capsules are pierced in the Podhaler and release spherical light porous particles with a high surface area and low density. Even in young children and children with reduced lung function, the powder is highly dispersible and reaches the infection sites. This Podhaler has advantages compared to conventional nebulizers: no need for electric power and portability. However, it seems to have a higher possibility for cough, dysphonia, and taste disturbances after inhalation.

The new field of nanomaterial even allows the creation of a totally new kind of therapy: Pilcer and colleagues combined two antibiotics on one particle: Tobramycin particles surrounded by a matrix composed of amorphous Clarithromycin. This allows the totally simultaneous delivery of the two antibiotics to the exact same site in the lungs (Pilcer *et al.*, 2013).

In the first 6 months of 2013, the Pubmed search platform shows nine original publications with the keywords “inhaled” and “tobramycin,” which shows how topical the development of nanomaterial-based biomedical applications in the field of CF is.

5.1.3 Cyclosporine Treatment of Bronchiolitis Obliterans after Lung Transplantations

The following information is summarized from (Onoue *et al.*, 2012) and (Behr *et al.*, 2009). Lung transplantation is an effective treatment of a variety of chronic end-stage lung diseases, and the subsequent immunosuppression is one of the key

interventions in those patients. Usually, a combined therapy with different systemic drugs is applied. One of the drugs used is cyclosporine A. Despite the different immunosuppressive drugs, common complications after lung transplantations are lung rejection and bronchiolitis obliterans. Bronchiolitis obliterans is thought to be induced by an ineffective epithelial regeneration and the histopathological pattern shows excessive fibroproliferation most probably because of injury and inflammation of the epithelial cells and subepithelial structures of small airways. The patients suffer a declined lung function (FEV1). It has been suggested that the mechanisms could involve T lymphocyte activation in the lungs. Systemically applied cyclosporine A blocks the T lymphocytes proliferation and reduces the activation. However, it is hypothesized that T lymphocytes invaded in the lung tissue may no longer be sufficiently suppressed. Thus, additional application of cyclosporine A in the lungs, reaching high concentrations in the lungs, could suppress T lymphocyte activation and prevent from developing bronchiolitis obliterans.

Liposomal cyclosporine A solution containing liposomes with a diameter of 50 nm can be inhaled with a customized electronic nebulizer that produces droplets with appropriate size for peripheral lung deposition. The liposomal cyclosporine A aerosol was very well tolerated by the patients without premedication. With two or three inhalations per day, the effective dose (5 mg) can be reached in the peripheral lungs. Inhaled liposomal cyclosporine A may be a promising new approach to optimize immunosuppression and thus to improve the long-term lung transplantation outcomes.

Cyclosporine A has not only immunosuppressive but also antifungal, anti-inflammatory, and antiparasitic properties and therefore may have preferable clinical outcomes for other diseases as well. Because of its low solubility and thus poor oral bioavailability, its low intestinal permeability and the systemic effects have, so far, limited the clinical use. Inhalable cyclosporine A (as a dry emulsion formulation) might also be used for treatment of asthma, airway inflammatory diseases, and other diseases (Onoue *et al.*, 2012).

5.1.4 Inhaled Chemotherapy in Lung Cancer

The following information is summarized from Zarogoulidis *et al.* (2012). Chemotherapeutic drugs

are usually administered intravenously and can cause adverse side effects. With a local drug delivery, which would deliver high drug concentrations to the target site, the side effects could be mitigated by preventing vital organs from being exposed to toxic drug concentrations in the systemic circulation. Several drugs, which have already been used for systemic treatments, have been successfully administered regionally in various types of cancer including advanced lung cancer. Even though it has been shown that the inhaled chemotherapy is a feasible way of treatment with a high potential for reducing adverse systemic side effects, further investigations about the pulmonary side effects are necessary.

5.2 Systemic Treatments via Inhalation

5.2.1 Inhaled Insulin for Diabetic Patients

The following information is summarized from Zarogoulidis *et al.* (2011) and Yaturu (2013). Diabetes is the global public health concern currently affecting more than 20 million people in the United States alone and the number of people suffering from this disease is predicted to increase in the future. The current state-of-the-art treatment is the insulin therapy with administration of insulin via subcutaneous injection. However, this method is invasive and requires three or more injections per day in type I diabetes patients. Therefore, new methods for administering insulin are being explored: artificial pancreas, transdermal insulin, buccal, oral, pulmonary, nasal, ocular, and rectal routes. One of the most promising routes and the preferred route by a majority of the patients is the pulmonary one. Exubera[®], an inhaled insulin product from Pfizer, has already been approved by the US Food and Drug Administration as well as by the European Medicines Agency and was on the market since August 2006. However, because of failure to gain market acceptance, the product was taken off the market in October 2007.

Using the pulmonary route, an aerosol of an insulin solution or dry powder insulin is released either mechanically or electronically by a device similar to an asthma inhaler. Inhalation of the released aerosols/particles brings the insulin to the bronchial tree and the alveoli. Compared to subcutaneous administration of insulin, the efficiency of insulin delivery via the pulmonary route

may be compromised by various factors, such as losses within the device, deposition in the throat and bronchial tubes, or incomplete absorption in the alveoli. However, considering those compromising factors already during the development of the device and the individual adjustment of the device, this can be overcome and the bioavailability usually reaches values between 10 and 46% depending on the product. The activity peak of inhaled insulin is measured after 60 min, which is similar to that of subcutaneous insulin. The amount of insulin, which needs to be taken, has to be adjusted to the body weight. Literature shows that the absorption of injected insulin is influenced—among other factors—by the body mass index (BMI). A finding that was not confirmed for inhaled insulin and that may make the inhaled insulin more reliable for obese diabetic patients than the subcutaneous delivery route. COPD, asthma, smoking, or other underlying comorbidities may alter the absorption of inhaled insulin. Specifically, when on exacerbation, the bioavailability may be reduced. A small number of studies showed that smoking increases the bioavailability that accentuates hypoglycemia. As smokers were excluded from most of the published studies, further investigations are needed. Upper respiratory infections in healthy persons have not shown any effect of the absorption of inhaled insulin. Inhaled insulin has an effect neither on lung lining composition nor on the protein composition of the lung lining fluid.

Taken everything together, using the inhalation route for the application of insulin has several advantages compared to subcutaneous injection of insulin: inhalation of insulin is noninvasive, does not cause injury, and exposes the body to approximately three times more insulin per dose than by injection.

5.2.2 Magnetic Resonance Imaging Contrast Agents

The following information is summarized from Santhosh and Ulrih (2013) and Mahmoudi *et al.* (2012). Magnetic resonance imaging (MRI) is a common tool to produce images of the human body for diagnostic purposes, for example, tumor diagnosis. Traditional techniques are not sensitive enough to detect small sized tumors and the traditional contrast agents are toxic, and therefore, it is difficult to deliver enough contrast agents into a

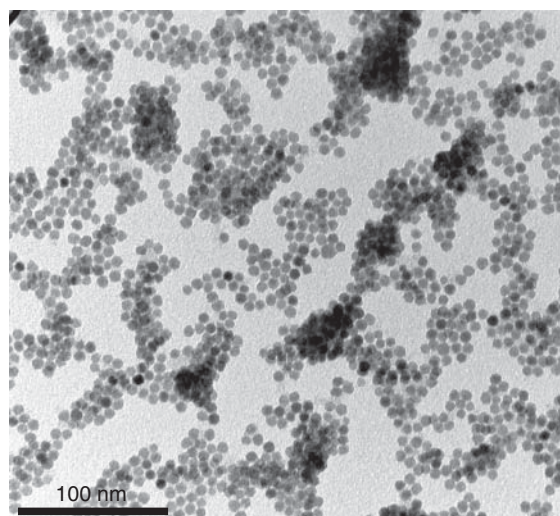


Figure 5. Transmission electron micrograph of superparamagnetic iron oxide nanoparticles obtained by the thermal decomposition method of an iron oleate precursor as described by Hyeon *et al.* (2002).

cancer cell. Iron oxide nanoparticles can be modified that they have a high sensitivity and targeting efficiency and therefore are optimal for use in MRI. They provide a better contrast and resolution because of their increased magnetic force. Superparamagnetic iron-oxide nanoparticles (SPIONs) (Figure 5) coated with organic molecules are commercially available as a contrast agent for MRI. They not only respond very strongly to the magnet field and thus improve the contrast and the resolution but also circulate for a longer time in the blood because they are densely packed with biocompatible polymers.

5.3 Specific Targets Not in Lungs: Magnetic Hyperthermia Treatment for Cancer

The following information is summarized from Santhosh and Ulrih (2013). The fact that increased temperature can kill cells is used in cancer therapy. So far, powerful radio waves were used to produce sufficient increase in temperature to destroy various types of carcinomas. However, this technique does not specifically target cancer cells and thus also affects the surrounding normal cells. The optimization of this method would be to only increase the

temperature in the cancer cells. The use of SPIONs makes this possible. SPIONs in the form of ferrofluids, which are modified specifically to target only the cancer cells, are injected. After the SPIONs reached the target cells, a magnet field and alterations in the direction of the magnet field are applied to stimulate the SPIONs to produce an enormous heat. The heat increases the temperature specifically in the target cells and either destroys them or makes them more susceptible to radiotherapy or chemotherapeutic treatment.

5.4 Future Potential and Considerations

As nanomaterials can be modified very specifically and even on an individual basis, the future potential of using nanomaterials for biomedical applications is huge. However, not all synthesis and loading processes were fully controlled and the optimization of the carrier—drug composite—has still to be defined. Therefore, a lot of research is currently carried out to investigate the opportunities and risks of nanomaterials. As nanosized objects behave different than objects based on the exact same bulk material, the risk of nanomaterials needs to be newly assessed. Most of the investigations are performed using *in vitro* approaches or *in vivo* studies, while human *in vivo* studies are mostly considered as ethically questionable. Besides finding the optimal characteristics of nanomaterial for the desired application as well as optimized production processes, the risk assessment is also the topic of numerous investigations, which will be one of the biggest challenges in the future. The interactions of the nanomaterials with components of the body, for example, proteins, cells, and tissues, are highly complex and not yet fully understood. With two recently published studies on animal experiments, we would like to highlight this complexity of the interaction with and the influences of nanomaterial on other cells and tissues. It was shown that (i) nanoparticle size plays an important role in influencing the activity of antigen-delivery systems in animal experiments (Stano *et al.*, 2012) and (ii) intranasally instilled polystyrene nanoparticles with a diameter of 20 nm in mice were captured preferentially by dendritic cells compared to bigger sized particles and that only the smallest particles induced enhanced antigen presentation to CD4⁺ T cells in local draining lymph nodes *in vivo* (Blank *et al.*,

2013). More investigations, especially about the risk of nanomaterials, are needed for a well-founded assessment of the risks and the opportunities of newly developed nanomaterials.

The inhalation pathway presents a noninvasive entry to the whole human body where also specific cells such as immune cells can be targeted and therefore is a promising route for future applications. As shown in this chapter, the field is not limited to targets in the lung; as the lung can be highly permeable, it can be used as a route to apply systemic or specifically targeted therapies. The future development and improvement of nanomaterials will allow for very specific targeting of single cell types and the directed release of drugs at specific sites.

6 SUMMARY AND CONCLUSION

Currently, a large amount of research is ongoing to explore new nanotechnological approaches to the design of novel inhalative nanomaterials for a controlled cell/tissue/organ targeting and a controlled release of a certain drug. Although many drugs for direct application via the lung, such as corticosteroids and antibiotics, are already available on the market, there is still a need for much more and complex investigations in the field of novel nanomaterials that can be used for inhalation. Taken altogether, the wide knowledge and the large number of recent studies on nanomaterial–lung interactions show the huge potential for the future use of specifically modified biomedical nanomaterials as a novel therapeutic approach via the inhalation route.

ACKNOWLEDGMENT

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END NOTES

- a. The first-pass effect describes the changes a drug undergoes until it reaches the systemic circulation. For most of the first-pass effects, the gastrointestinal lumen and enzymes (gut wall, bacterial, and hepatic) are responsible.

- b. Half-life is the time needed for half of the molecules deposited into the lungs to disappear from the tissue.

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Nanomedicine for the Brain and the Eye: Disease Management in Poorly Accessible Compartments of the Body

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1 INTRODUCTION

1.1 Nanotechnology to Reach Poorly Accessible Compartment of the Body

The advent of nanodrugs allows newer strategies to treat poorly accessible lesions, such as those affecting the central nervous system (CNS) or the posterior pole of the eye. These compartments of the body are protected by biological barriers. Their selectivity was first described around 1930 (Figure 1), and it was finally stated that hydrophobicity and low molecular weight are requested features of putative drugs for the CNS.

Four decades later, techniques for assembling lipids in liposomes were developed. Liposomes interact with biological membranes and carry molecules into the cytoplasm. The partially successful attempt of exploiting liposomes as carriers of drugs to treat brain tumors followed. It was

soon clarified that, in neurology, only nanoliposomes retained both biocompatibility and efficacy as drug carriers and adjuvants (Schackert *et al.*, 1989). These experimental evidences together with the bloom of nanotechnology have stimulated researches on new nanomaterials for the diseases of the CNS. Nanotechnology may also target the retina, another site protected by a biological barrier named the blood–retina barrier (BRB).

1.2 Biological Barriers Protect the Central Nervous System

Because of its poor accessibility, the CNS may require invasive manipulations for diagnosis and treatment. The skull and the spinal canal protect it with a bone shell that cannot be expanded, exacerbating the symptoms in case of edema, bleeding, or other expansive lesions, such as tumoral or fungal

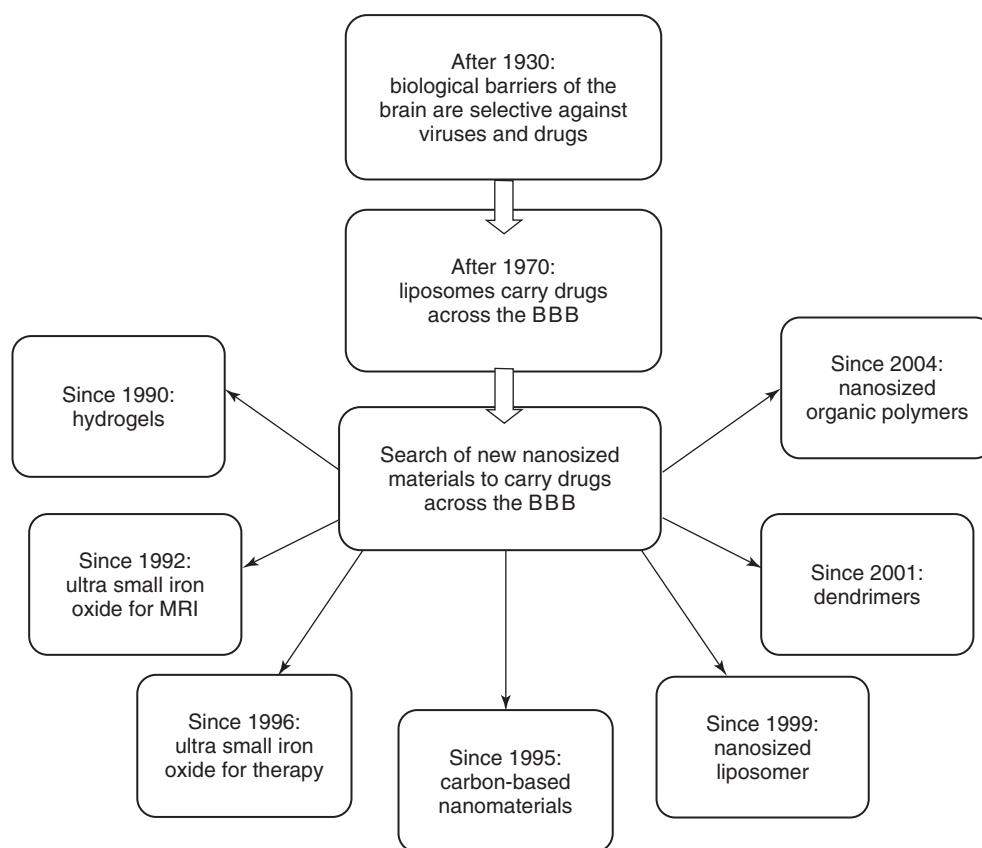


Figure 1. Footsteps through the blood–brain barrier: from its discovery to the development of drugs and nanomaterials able to bypass it.

growth. In addition, the CNS is segregated from the other regions of the body by the presence of two biological barriers, the blood–brain barrier (BBB) and the blood–cerebrospinal fluid barrier (BCSFB). Both are highly selective, preventing most molecules from reaching the brain. BBB modulates the diffusion from blood to CNS and the BCSFB from CNS to cerebrospinal fluid. The embryonic development and most relevant physiological mechanisms featuring the brain barriers have been recently reviewed (Nico and Ribatti, 2012; Siegenthaler and Pleasure, 2011; Saunders, Liddelwand, and Dziegielewska, 2012; Stolp *et al.*, 2012).

BBB diffusely develops along the cerebral vessels, in the endothelia and in glia, astrocytes, and pericytes, in contact with it (Figure 2). Its selectivity is based on a variety of mechanisms, spanning from energy-dependent transport to transcytosis, whereas simple diffusion processes are very limited. The multidrug resistance protein 1, MDR1 is a

widespread efflux system for drugs (Schinkel *et al.*, 1994). The BCSFB is localized in adults in the ependymal cells, at the inner (ventricular) and outer (meningeal) surfaces of the nervous parenchyma and in the choroid plexus. Its degree of selectivity to xenobiotics and autologous compounds is much lower than that of the BBB, mainly based on the multidrug resistance-associated protein MRP1 (Baehr, Reichel, and Fricker, 2006; Sun *et al.*, 2003) (Figure 3). The most important trait of a fully functional barrier is the development of the tight junctions between cells and the expression of their featuring markers. The endothelial cells of the BBB are polarized, and come in close contact with the glial components. The rich net formed by the feet of astrocytes keeps the normal neural architecture in place and connects the neurons with the endothelia, whereas the pericytes are discontinuously interposed between the endothelia and the astrocytes (Redzic, 2011).

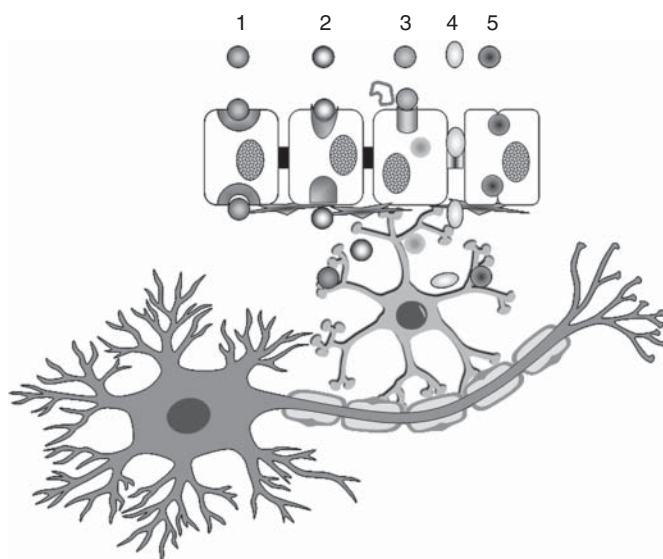


Figure 2. Scheme of the blood–brain barrier (BBB). An astrocyte (at the center) bring together a neuron (lower cell) and the endothelial cells (upper layer), surrounded by several pericytes (star-like cells). The endothelial cells are joined by tight junctions (Tj, black intracellular rectangles) and are polarized. Their outer face is exposed to the blood and to substances dissolved in it. In the scheme, five different mechanisms for nanoparticles transport are represented. From the left: (1) transport-mediated transcytosis; (2) active transport; (3) substrate modification; (4) paracellular diffusion across the Tj; and (5) simple transcytosis.

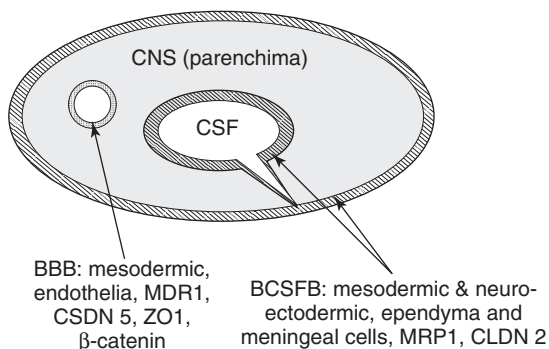


Figure 3. Scheme of the blood–brain barrier (BBB) and the blood–cerebrospinal fluid barrier (BCSFB). The marker of tight junctions in BBB, CLDN5, is present in high concentrations, whereas the content of CLDN2 is low in the BCSFB. CLDN, claudin; CNS, central nervous system; CSF, cerebrospinal fluid; MDR1, multidrug resistance protein 1; MRP1, multidrug resistance-associated protein 1; ZO1, zone occluding 1.

The ability of nanoparticles (NPs) to cross the BBB was a postulate not completely documented by experimental evidences, at least *in vivo*. In many cases, after systemic administration, NPs accumulated into the vascular bed around the lesion and promoted sustained release of drugs, without crossing the barrier. On the other side, the successful

attempts to force NPs into the targeted cells, mainly obtained *in vitro*, require careful refinement before clinical exploitation: the metabolic fate of depleted carriers should be known in details, to avoid unpredictable toxicity in tissues equipped for limited metabolic processing and clearance of xenobiotics (Boyes *et al.*, 2012). In mice, even the apparently biocompatible liposomes showed relevant toxicity, when formulated at large sizes (Schackert *et al.*, 1989).

1.3 Barrier Properties Are Modified in Tumors and Other Diseases

Specific alterations of the barrier occur in the region, anatomically consistent with the BBB, surrounding focal malignancies developing in the brain, irrespective from their metastatic or primitive origin. The term *blood–tumor barrier (BTB)* was introduced to indicate this barrier, characterized by diffuse presence of pores as large as 12 nm in diameter (Sarin *et al.*, 2009) and apoptotic aspects in endothelia (Molnár *et al.*, 1999). Despite its enhanced permeability to contrast media (Percy *et al.*, 2011) and to cells (metastatic and macrophages), the BTB prevented free diffusion of antitumor agents,

eventually limiting the efficacy of conventional treatment. A number of strategies and methods apt to increase the permeability of the BTB were therefore developed (Kato, Tsuchida, and Kawamoto, 2005; Groothuis, 2000).

Genetic, neurodegenerative, inflammatory, and autoimmune diseases diffusely affect the CNS and its cellular types. The mechanisms often involve abnormal anatomic relations between endothelia and pericytes and possible lack of tight junctions, which in turn increases the risk of intracerebral hemorrhages (Daneman *et al.*, 2010; Nicolas *et al.*, 2013). Multiple sclerosis and acute ischemia have in common the alterations of tight junctions, basal lamina, and adherence of the feet of the astrocytes to the endothelium. The vascular alterations occurring in Alzheimer disease reduce the cerebral flux at a lower level than in acute ischemia, but the causative defect is still unknown (Krol *et al.*, 2013). A certain degree of leakage of the BBB is present also in infectious diseases of the CNS, sustained by viruses, prokaryotes, fungi, and parasites.

1.4 The Blood–Retina Barriers and the Posterior Pole of the Eye

The easiest way of pharmacological delivery to the eye remains the topical application of drugs and ointments to the anterior part of the organ. The

permeability to drugs of the different ocular layers, as measured in *in vitro* experiments, was found to be higher for the conjunctiva followed by the sclera and the cornea (Loch *et al.*, 2012). Instead, the posterior pole is barely accessible through this way: from out- to inside, three layered tissues (sclera, choroid, and retina) come in succession. Not surprisingly, the retina, a tissue sharing with the brain the embryological origin and the neural nature, is another tissue tightly protected by biological barriers, the inner and the outer blood–retina barriers (i/oBRBs). The former regulates the exchanges from the blood in retinal vessels to retina; the latter from the blood in choroid to retina; the responsible cells are those of the vascular endothelium of small retinal vessels and of the pigmented epithelium (Figure 4) (Schlosshauer, 2007; Klaassen, Van Noorden, and Schlingemann, 2013). Barriers with fully functional tight junctions express complex associations of claudins, their main markers, whereas the transporters of drugs mainly belong to the organic anion transporting (OAT) polypeptides. Though different for its structure and ontogeny, the BRB is functionally similar to BBB (Hosoya and Tachikawa, 2009; Kim *et al.*, 2006; Larsen *et al.*, 2009; Steuer *et al.*, 2005). Main ways for drug delivery to the eye are summarized in Figure 5. Among them, only the intravitreal injection or implant seems to provide effective amounts of drugs to the retina, and nonetheless the penetration into

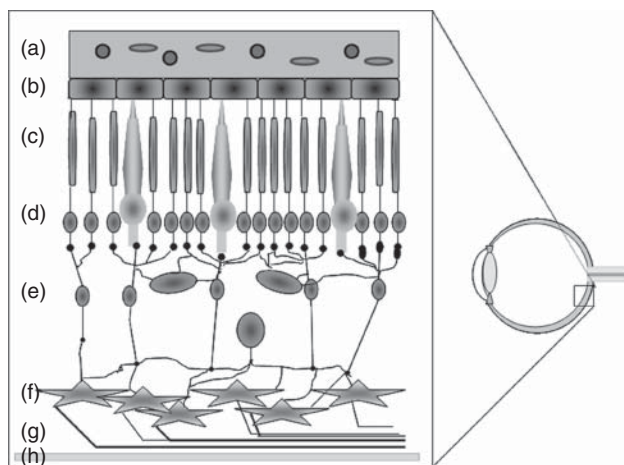


Figure 4. Section of human eye (on the right) and enlarged schematic representation of layers in the posterior pole of the ocular globe (on the left). (a) vascularized choroid (outer endothelial BRB); (b) pigmented epithelium (outer epithelial BRB); (c) cones and rods and (d) their nuclei; (e) nuclei of bipolar (smaller), horizontal (upper), and amacrine (lower) cells; (f) ganglion cells; (g) nerve fibers; (h) inner limiting membrane layers (inner BRB in vascular endothelia).

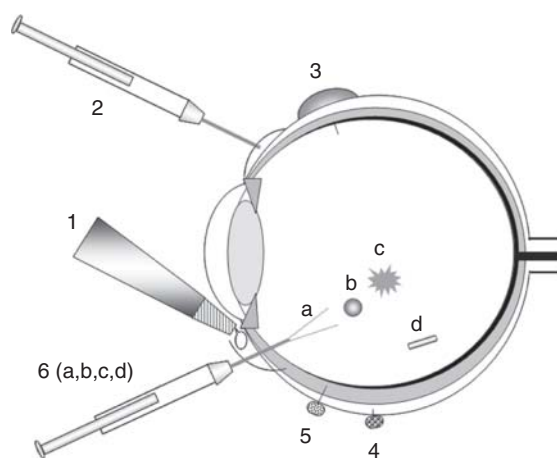


Figure 5. Route of drug administration to the eye. (1) topical; (2) sub-conjunctiva injection; (3) episcleral permanent implant; (4) intrasceral depot through micro needle; (5) choroidal injection through micro needle; (6) intravitreal injection: (a) solution, (b) biodegradable reservoir, (c) suspension, (d) free-floating implant.

the eye is associated with side effects as serious as endophthalmitis, whereas glaucoma or cataract are frequent long-term complication of sustained and prolonged (some years) intravitreal release of corticosteroid or anti-inflammatory drugs (Edelhauser *et al.*, 2010).

2 EXPERIMENTAL MODELS FOR CNS AND EYE

2.1 *In Vitro* Models for CNS

This section spans from simple unicellular systems, like those used in preliminary studies in neuro-oncology, to 3D and multicellular cultures, mimicking the complex structure of BBB and neural parenchyma. *In vitro* studies in neuro-oncology most frequently implemented single-layer cultures of human and murine lineages of neurological tumoral cells, such as those derived from glioma (GL261, C6, F98), glioblastoma multiforme (U-251, U-87; DBTRG, LN229, U118), and astrocytoma (T98G) (Aoki *et al.*, 2004; Chang *et al.*, 2012; Firth, Oliver, and McKernan, 1984; Lu *et al.*, 2012b; Madhankumar *et al.*, 2006; Molinari *et al.*, 2007; Tang *et al.*, 2011).

To overcome the limits of monolayer cultures, in which cells are equally exposed to substances

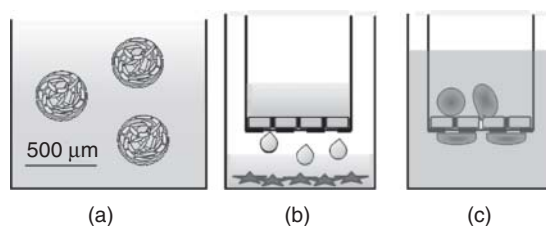


Figure 6. Methods for studying *in vitro* the effects of nanoparticles on the CNS and neuronal cells. (a) Neuronal precursors growing as 3D-microspheres in a poorly adhesive environment. (b) Transwell system. A monolayer of endothelial cells is grown on the upper surface of a semipermeable membrane. The cells derived from the CNS (glial cells, astrocytes, and neuronal) are grown in the inferior chamber. The mature endothelia develop normal Tj. Their upper surface represents the blood side, the lower the brain side. The cells derived from the CNS types could be grown in a single layer of identical cells, or in layers stratified, each containing different cellular types. (c) Transwell migration assay. The upper chamber contains migrating cells, such as stem cells or macrophages. After migration, the cells are recorded on the lower side of the transwell membrane.

dispersed in the medium, a 3D model was proposed. Human embryonic stem cells, previously differentiated into Pax6+ cells, were grown in a medium allowing poor adhesion, until spheres of about 500 μm were obtained (Figure 6a). These spheres were proposed as a model for studies on developmental neurotoxicity of NPs, because of their sensitivity to specific toxicants (Hoelting *et al.*, 2013). The BBB, absent in these spheres, was experimentally reproduced in confluent monolayer of cultured brain endothelial cells, which express markers of fully developed tight junctions and retain the main mechanisms of transport for xenobiotics. In this case, the missed components are the glia and the neuronal targets (Qiao *et al.*, 2012).

The transwell model is a more complete variant, in which the brain endothelium grows to a confluent monolayer on the upper side of the semipermeable membrane dividing a Boyden chamber. The complete functionality of the BBB was documented by the expression of claudin 5, β -catenin, and ZO1, the main markers of tight junctions (Brun, Carrière, and Mabondzo, 2012), or by an average transendothelial electrical resistance of ca 150–200 Ωcm^{-2} (Nair *et al.*, 2012). Thanks to the polarization of these cells, the upper space could be compared to the blood, and the lower chamber to the parenchyma compartment of the CNS, with the typical cells, neuronal, glial, or both, grown at its

bottom (Figure 6b). The test NPs are added to the medium filling the upper chamber, and the ratio across the transwell and the effects on the cells in the lower space are measured. Stem, tumoral cells, and macrophage are able to cross the BBB in the transwell migration assay (Figure 6c) and respond to nanoparticles (NPs) (Tao *et al.*, 2012, Zhang *et al.*, 2012). An innovative 3D structure of the brain matrix was recently proposed. A nanoporous hydrogel of hyaluronic acids was functionalized with the RGD (arginine-glycine-aspartic acid) tripeptide: malignant glioma cells invaded it with modalities similar to those seen in the brain affected by the tumor. The speed and entity of the spread depended on the stiffness of the matrix and the density of the tripeptide (Ananthanarayanan, Kim, and Kumar, 2011).

2.2 *In Vivo* Models for CNS

In vivo models in neuro-oncology include intracranial inoculation of malignant cells in rodents and tumors spontaneously developing in selected strains of animals. The subcutaneous implants, which lack the BBB, can be criticized (Arosarena *et al.*, 1994). Different considerations should be applied to the study of neurodegenerative diseases, as experimental models of rodents with genetically or chemically induced diseases are available. The subcutaneous injection of the human proteolipid protein peptide139–151 with adjuvant induces in rats the autoimmune encephalomyelitis, which reproduces multiple sclerosis. (Millward *et al.*, 2013). A neurodegenerative conditions similar to the Alzheimer's disease develops in APP/PS1 transgenic mice, overexpressing the mutant precursors of amyloid (APPK670N/M671L) and of presenilin (PS1M146L) proteins, associated with the deposition of the amyloid plaques. In these animals, the intravenous (i.v.) injection of PEG-USPIO (polyethylene glycol–ultrasmall superparamagnetic iron oxide) conjugated to the peptide A β 1-42 produced spotted signal at the magnetic resonance imaging (MRI) only in the presence of amyloid plaques and independent from altered BBB permeability (Wadghiri *et al.*, 2013). The inflammatory responses of the pericytes and the following damage to the brain endothelia were instead precociously present in mice knocked out for all genes codifying for the APOE (apolipoprotein E) proteins and

in mutants expressing the APOE4, the condition associated to the development of the Alzheimer's disease in humans (Carmeliet and De Strooper, 2012; Bell *et al.*, 2012).

2.3 Experimental Models for the Posterior Pole of the Eye

Established methods of *in vitro* study include culturing cells of the arising retinal pigment epithelium (ARPE-19), retinal ganglion cells (RGC-5), human retinoblastoma, or retinal micro vascular endothelial cells. As for the CNS, these methods lack the retinal architecture and the contemporary presence of components, barrier, and parenchyma (Del Pozo-Rodríguez *et al.*, 2008; Guo *et al.*, 2013a,b; Jo *et al.*, 2012). Explants of retina maintained *in vitro* preserve the normal retinal architecture only for a short time, soon after early degenerative and apoptotic markers develop. A recently published method proposed an array of nanotubes of TiO₂ to enhance their survival up to 14 days. The comparison with slices of nervous tissue put into evidence that the optimal performance strictly depended on the type of tissue, physical chemistry and nature of the array, and especially from its mechanical and hydration properties, which were mainly responsible for the longer survival (Dallacasagrande *et al.*, 2012).

Experimental studies *in vivo*, mostly performed in anesthetized rats, included various techniques of implant and inoculation of nanostructured devices or NPs, such as injection in the subretinal space (Han *et al.*, 2012) or in the vitreous humor (Akiyama *et al.*, 2012). One eye was used for treatment, the contralateral as control; at the term of planned study time, the animals were sacrificed and both eye enucleated for measuring and comparing the effects. Topical instillation of eye drops to reach the posterior pole is poorly performing and therefore this way of administration was only occasionally tested in mice and rabbits (Araújo *et al.*, 2011, 2012; Khan *et al.*, 2012). For a recent review, see Del Pozo-Rodríguez *et al.* (2013). Enucleated eyes of rats were mounted in a Franz-type diffusion cells for studies completely performed *ex vivo* (Araújo *et al.*, 2012).

2.4 Clinical Trials

Clinical studies with nanodrugs in neuro-oncology and in neurodegenerative diseases are limited by

epidemiological and ethical considerations, including the low prevalence of tumors and the severity of prognosis. Similarly, for the posterior pole of the eye, only endovitreous implants of nanoporous silica delivering steroids have been approved until now for clinical use. The prevalence of late complications within the 3-year period of evaluation was high, up to 100 and 40% for cataract and glaucoma, respectively, probably because of too sustained and prolonged release of steroids (Callanan *et al.*, 2008; Goldstein *et al.*, 2007; Edelhauser *et al.*, 2010). For the brain, as for the eye (Edelhauser *et al.*, 2010), the need for further studies is evident, and protocols with the highest level of evidence [scoring scale according to Haase and Chung (2011)] are especially expected.

3 NANOTECHNOLOGY CAN BE APPLIED TO NEURO-ONCOLOGY

3.1 Lipidic Nanoparticles

Like traditional formulation of drugs, NPs, including liposomes and micelles, are able to cross the BBB thanks to their hydrophobicity (Escuder-Gilabert *et al.*, 2004; Hills, 1992; Kai *et al.*, 1996; Lessigiarska *et al.*, 2005). Liposomes were the earliest nanocarriers to be considered in neuro-oncology. Shortly preceded by studies in tumor cell lines (Firth, Oliver, and McKeran, 1984), the liposomal approach was firstly proposed for clinical use by McKeran *et al.* (1985). Before, antitumoral drugs were given by intrathecal injection, but this procedure did not work well, because of the too rapid clearance possibly due to the high blood flow at the tumor site. Preliminary experiments on *in vitro* cultured glioma cells supported the efficiency of vincristine- and bleomycin-loaded liposomes (Firth, Oliver, and McKeran, 1984). Further investigation in a phase 1 clinical trial demonstrated that the intrathecal injection of charged liposomes was well tolerated. In fact, after administration, the peak of the drug in the peripheral blood was lower and shorter, and the urinary clearance was much lower than that of the free drug, warranting a longer half-life at the site of action (McKeran *et al.*, 1985). The size of the anionic liposomes, however, was not specified by the authors, but, as the liposomes were prepared according to Skoza and Papahadjopoulos (1978), they should have been sized 100–200 nm.

In this earlier phase, the attention was focused more on the lipid composition than on the liposome size: nanotechnology was not the matter. An anticipating paper claimed that larger multilamellar liposomes ($>5\text{ }\mu\text{m}$) unsafely accumulated in the brain, whereas the smaller ones (40–80 nm) were better tolerated (Schackert *et al.*, 1989). Other studies favorable to the use of liposomal antineoplastic drugs followed (Boiardi *et al.*, 1999; Khalifa *et al.*, 1997; Sharma *et al.*, 1997; Siegal, Horowitz, and Gabizon, 1995). However, only after the year 2000, antineoplastic agents embedded in nanosized liposomes, registered and approved for use in humans, appeared in the neuro-oncology clinical research. Daunorubicin embedded in 45 nm liposomes (Daunoxome[®]) and doxorubicin in pegylated liposomes (Caelyx[®], 80 nm) were given intravenously to patients affected by malignant tumors of the brain. Both formulations performed well in terms of sustained release, biodistribution to the tumoral tissue, tolerability, and the overall survival time of treated patients (Albrecht *et al.*, 2001; Fabel *et al.*, 2001; Koukourakis *et al.*, 2000). At present, three are the active antineoplastic drugs, embedded in liposomes, which play the most important role in clinical neuro-oncology: doxorubicin, daunorubicin, and cytarabine, also called *ara-C* (Tables 1 and 2). The liposomal (and pegylated liposomal) doxorubicin and daunorubicin were well tolerated and performed well. They were effective in reducing progression and symptoms of a number of primitive and metastatic solid tumors affecting the CNS. The metastatic diffusion of the epithelial ovarian cancer seemed to be resistant to this formulation (Kasprowicz *et al.*, 2008). The liposomal formulations appeared to be safer than soluble drug, especially for the cardiac complications. Liposomal (and pegylated liposomal) cytarabine worked well for the treatment of primitive tumors of the CNS, for the metastatic localization in the CNS of the breast cancer, and for the cerebral localization of leukemia or lymphoma. Because this drug was charged, following the necessary intrathecal administration, by significant neurotoxicity, the concomitant prophylactic use of dexamethasone became the recommended routine (Benesch *et al.*, 2009; Peyrl *et al.*, 2009). The association of the newer liposomal drugs with other adjuvant therapies, such as radiotherapy, antineoplastic drugs, or drugs for the control of emesis and cerebral edema, was generally safe and helpful, with only a few exceptions (Hau

Table 1. Clinical studies with liposomal and pegylated liposomal daunorubicin and doxorubicin administered intravenously for the treatment of brain tumors.

Tumor	Associated adjuvants	Outcome	References
AG	Tamoxifen	Moderate	Hau <i>et al.</i> (2004)
GBM, MB	Radiotherapy	Positive	Boiardi <i>et al.</i> (1999)
GBM, TRT	Carboplatin plus etoposide	Positive	Fiorillo <i>et al.</i> (2004)
GBM	None, surgery, temozolomide, novantrone	Positive	Zucchetti <i>et al.</i> (1999), Fabel <i>et al.</i> (2001), Boiardi <i>et al.</i> (2003, 2005)
GBM	Temozolomide	Moderate	Chua <i>et al.</i> (2004)
GBM	Radiotherapy, temozolomide	Tolerated	Ananda <i>et al.</i> (2011), Beier <i>et al.</i> (2009) ^a
GBM, choroid plexus carcinoma	Topotecan	Positive	Wagner <i>et al.</i> (2008)
Disseminated meningioma	Chemotherapy (various schemas)	Moderate	Chamberlain and Glantz (2005)
NET, TRT, MB, other	Dexamethasone, ranitidine, diphenhydramine	D _{max} : 60 mg m ⁻²	Marina <i>et al.</i> (2002)
Recurrent brain tumors	—	Positive	Lippens (1999)
Recurrent clivus chordoma	Thalidomide	Positive	Schönegger <i>et al.</i> (2005)
Metastatic cancer (breast, vulva, other)	Paclitaxel, thalidomide, temozolomide	Positive	Schwonzen, Kurbacher, and Mallmann (2000), Huang <i>et al.</i> (2002), Caraglia <i>et al.</i> (2006)
Metastatic ovarian cancer	Radiotherapy, paclitaxel	Ineffective	Kasprowicz <i>et al.</i> (2008)

AG, anaplastic glioma; D_{max}, maximum dose (in children); GBM, glioblastoma multiforme; MB, medulloblastoma; NET, CNS primitive neuroectodermal tumor; TRT, atypical teratoid rhabdoid tumor.

^aRegistered at the ClinicalTrials.gov (NCT00944801).

Table 2. Clinical studies with liposomal cytarabine administered intrathecally for the treatment of brain tumors.

Tumor	Associated adjuvants	Outcome	References
MB, GCT, NET, AE, AO, TRT, RPM	Dexamethasone ^a	—	Benesch <i>et al.</i> (2009)
Meningeal diffusion of A, grade II	Temozolomide	Positive	Passarin <i>et al.</i> (2010)
NET, TRT	Dexamethasone	Positive	Peyrl <i>et al.</i> (2009)
E	Polychemotherapy and dexamethasone	Positive	Lassaletta <i>et al.</i> (2007)
Meningeal diffusion of MB, NET, E and other primitive tumors	Chemiotherapy and radiotherapy, desamethasone	Positive ^b	Navajas <i>et al.</i> (2012)
Meningeal metastasis	—	Neurotoxicity	Chamberlain (2012)
Metastasis of breast cancer	Temozolomide and/or radiotherapy	Positive	Glas <i>et al.</i> (2008), Hoffmann, Buhk, and Strik, (2009), Gaviani <i>et al.</i> (2009)
Prophylaxis of acute lymphatic leukemia dissemination	Polychemotherapy and dexamethasone	Neurotoxicity	Jabbour <i>et al.</i> (2007)
CNS leukemia (relapse)	Radiotherapy, dasatinib	Positive	Aichberger <i>et al.</i> (2007), Seif, Reilly, and Rheingold (2010)
Intracranial B-cell lymphoma	Polychemotherapy, prednisone, and granulocyte-colony stimulating factor	Positive	Falchi <i>et al.</i> (2010)

A, astrocytoma; AE, anaplastic ependymoma; AO, anaplastic oligodendroglioma; CNS, central nervous system; E, ependymoma; GCT, germ cell tumor; NET, CNS primitive neuroectodermal tumor; MB, medulloblastoma; RPM, rhabdoid papillary meningioma; and TRT, atypical teratoid rhabdoid tumor.

^aCautious use recommended.

^bRetrospective evaluation.

et al., 2004; Ananda *et al.*, 2011; Chua *et al.*, 2004). Very few clinical trials with liposomal antineoplastic molecules other than daunorubicin, doxorubicin, and cytarabine are ongoing, namely those using vincristine, irinotecan, and lomustine. As far as it concerns the nanocarriers, only two innovations are present at the ClinicalTrials.gov registry, the pegylated liposomal doxorubicin conjugated with glutathione (GSH) and the albumin NPs. The former were targeted to the GSH transporters present on the BBB (trials NCT01386580 and NCT01818713), whereas albumin NPs (Abraxane®, NCT00738361) were reserved to the treatment of metastatic, unresectable uveal melanomas, spread to multiple organs, CNS among them. All the registered clinical trials are in an early clinical phase, I or II (Table 3).

The living research on nanoliposome pharmacology provides a number of innovative approaches, which still remain confined to the laboratory. The conjugation of NPs at the surface with the interleukin-13 represented a further improvement to address the system to the cells of the glioblastoma, an elusive, nonfocal tumor that selectively expresses the molecular receptor for interleukin-13. The selective targeting to glioma cells was experimentally proved *in vitro* and *in vivo* (Madhankumar *et al.*, 2006). Other therapeutic strategies that were developed against gliomas implemented the use of liposomes, such as the therapy with monoclonal antibodies (Chen and Mitchell, 2012), the photodynamic therapy (Molinari *et al.*, 2007), and the boron neutron capture therapy (Doi *et al.*, 2008; Nakamura, 2009; Yanagië *et al.*, 2008). Tests *in vitro* or *in vivo* demonstrated that the liposomal drugs provided, in comparison with traditional formulations, higher bioavailability, optimized pharmacology, and improved cytotoxic effect against glioblastoma cells or experimental intracranial tumor in rodents. The efficiency of nanolipids as anticancer carriers to the CNS greatly improved if combined with the convection enhanced delivery, a technique aimed to enhance the bioavailability at the tumor site (Grahn *et al.*, 2009; Saito *et al.*, 2004, 2006). Liposomes designed to release their content at a moderate hyperthermia limited the cytotoxicity to the neoplasia (Aoki *et al.*, 2004; Kakinuma *et al.*, 1996).

In addition to their value as carrier for drugs, the liposomes were also suitable systems for therapy with genetic material. Liposomal formulations successfully assisted the transduction of both DNA and siRNA. The expression of the interferon beta

gene was enhanced in a small sample of patients with malignant gliomas (Yoshida and Mizuno, 2003; Yoshida *et al.*, 2004). Instead, the transduction of siRNA was tested *in vitro* and *in vivo* in rodents: it downregulated a protein repairing the pharmacologically induced damage to the DNA of the glioblastoma cells, so that resistance to the conventional therapy developed (Kato *et al.*, 2010).

3.2 Polymeric Organic Nanomaterials

The expectation that liposomes could cross the BBB was not supported by experimental evidences: the only demonstrated ability of liposome formulations was that they accumulated in the vascular bed surrounding the tumor and promoted sustained release of the active drug in this limited region with lower systemic toxicity.

This partial failure strongly incentivized further studies or the design of modular and multifunctional nanomaterials specifically addressed to BBB transporters and to tumor cells. Loading polymeric nanocarriers with paramagnetic or optical probes enhanced the MRI. It was also possible to guide magnetic nanocarriers to the target, by locally applying a magnetic field. These “smart” NPs simultaneously provide locally implanted sensor, for repeated imaging and follow-up, and promote on-demand release of active molecules (Cabane *et al.*, 2012; Ling *et al.*, 2012; Tong *et al.*, 2012; Wanakule and Roy, 2012). The design of these NPs can be very complex. In the work of Wang *et al.* (2012a), a polymeric core was inserted into pegylated liposomes, whose surface was functionalized with peptides [TAT (transactivator of transcription) and RGD (arginylglycylaspartic)] that improve BBB crossing and gene condensation. These multimeric nanoshells were loaded with magnetic crystals and active principles (inhibitory genes or antineoplastic drugs) against malignant gliomas. Every component of the nanoshell was necessary to maximize the effect on intracranial glioblastoma in rats (Wang *et al.*, 2012a). Other works applied similar strategies, decorating the surface of polymeric NPs with peptides or protein to facilitate the passage through the BBB (Georgieva *et al.*, 2012; Chang *et al.*, 2012).

Among the carbon-based nanomaterials, a metallofullerol, $[\text{Gd}@\text{C}_{82}(\text{OH})_{22}]_n$ (Figure 7a) has inhibited tumor growth *in vitro* and *in vivo* (Yim *et al.*, 2012). A single layer of graphene

Table 3. Clinical trials, exploiting nano-formulation of anticancer drugs for brain or meningeal tumors or metastasis, deposited at the ClinicalTrials.gov repository (<http://clinicaltrials.gov>).

NCT	Start date	End date	Disease	Administration	Formulation	Type/phase	Country
00944801^a	July 2002	May 2009	GBM	i.v.	Caelyx[®]	Interventional I/II	Germany
00734682	August 2008	NR	GBM	i.v.?	Irinotecan [®]	Interventional I	USA
00769093	October 2008	December 2013	GBM, recurrent	i.v.	Ferumoxyl	Diagnostic I	USA
01044966	September 2009	September 2014	GBM, recurrent	Intrathecal	DepoCyt [®]	Interventional I/II	USA
00019630	July 1999	NR	Brain neoplasm in children	i.v.	Liposomal doxorubicin	Interventional, I	USA
01222780	September 2010	December 2013	Neuroblastoma, brain lymphoma, or metastasis	i.v.	Marqibo [®]	Interventional, I/II	USA
01386580	July 2011	December 2013	Brain metastasis, recurrent malignant glioma	i.v.	2B3-101	Interventional, I	EU
00465673	September 2005	July 2009	Brain metastasis (breast)	i.v.?	Lipo-Dox[®]	Interventional, II	Singapore and USA
00801216	January 2007	January 2012	Metastatic B-cell lymphomas	Intrathecal	Depocyt [®]	Interventional, II	Italy
00854867	February 2011	October 2012	Brain metastasis, neoplastic meningitis	Intrathecal	Depocyt[®]	Interventional, I	Austria
01563614	March 2012	March 2015	Leptomeningeal metastasis (melanoma)	Injection (near the spinal cord)	Liposomal cytarabine (and lomustine)	Interventional, I	Germany
01818713	March 2013	April 2014	Metastatic meningeal carcinoma	i.v.	2B3-101	Interventional, II	EU
00992602	April 2011	NR	Brain metastasis (breast)	Intrathecal	Depocyt [®] and methotrexate	Interventional, II	USA
00738361	August 2008	August 2012	Intraocular melanoma	i.v.	Abraxane [®]	Interventional, II	USA

2B3-101, glutathione pegylated liposomal doxorubicin; Abraxane[®], nano-albumin paclitaxel; Caelyx[®], pegylated liposomal doxorubicine; Depocyt[®], liposomal cytarabine; Irinotecan[®], nanoliposomal CPT-11; Lipo-Dox[®], liposomal doxorubicin; and Marqibo[®], liposomal vincristine; GMB, glioblastoma; NR, not registered. NCT: Entry number at the ClinicalTrials.gov. In bold: trials registered as completed, ^aone of them has published results (doi: 10.1186/1471-2407-9-308).

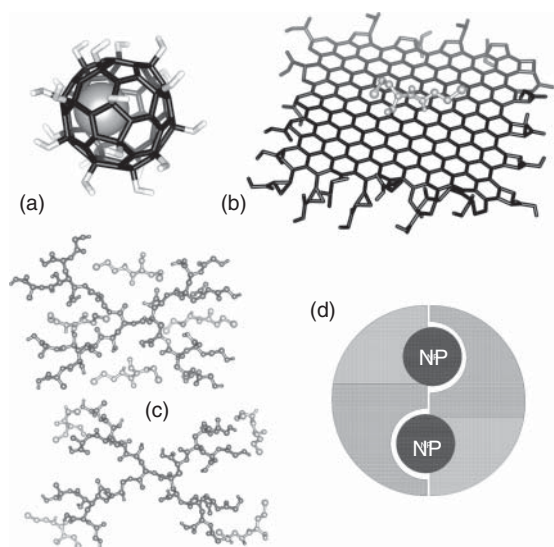


Figure 7. Graphical representation of different types of nanomaterials exploited for use in studies of the CNS. (a) Metallofullerol similar to the antitumoral agent $[\text{Gd}@\text{C}_{82}(\text{OH})_{20}]_n$. (b) Graphene functionalized with acrylic acid and loaded with the antineoplastic agent BCNU. (c) Dendrimers with inner (top) and outer (bottom) functionalization. (d) Silica nanosphere loaded with USPIO, for long-term magnetic resonance imaging. [Molecular graphics images were produced using the UCSF chimera package from the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco, supported by NIH P41 RR001081 (Pettersen *et al.*, 2004).]

oxide with hydrophilic surface functionalization was loaded with the antineoplastic drug BCNU (1,3-bis(2-chloroethyl)-1-nitrosourea) (Figure 7b) and tested *in vitro*. This system significantly prolonged BCNU half-life by lowering its thermal instability and dramatically improved its efficiency, as demonstrated in cultured glioma cells (Lu *et al.*, 2012b).

The dendrimers can conjugate probes and active drugs. The functionalization of their reactive groups, localized at the surface or inside the dendrimer (Figure 7c), leads to the acquisition of new properties. The availability of biocompatible dendrimers opened new insights: after i.v. administration to rodents affected by gliomas, dendrimers sized 11.7–11.9 nm, efficiently crossed the barrier and accumulated in the tumoral cells. The site of accumulation was precisely detected by magnetic resonance imaging using dendrimers functionalized with superparamagnetic and fluorescent probes (Sarin *et al.*, 2008). Similarly, dendrimers linked

at their surface with Angiopep-2, a 19-amino acid peptide-derived aprotinin, showed high concentration in the brain parenchyma. The functionalization with an optical probe allowed localizing them mainly in selected regions of the brain (Gao *et al.*, 2013). Moreover, boronated dendrimers were conjugated with monoclonal antibodies for the immunotherapy of gliomas (Chen and Mitchell, 2012).

While works in this field are important for number, quality, and complexity, the way of theranostic for the neuro-oncology has still to begin. Necessary improvements are required to ensure better bioavailability, long-term efficiency, and ease in administration and manipulation.

4 NANOMATERIALS WITH HIGH VERSATILITY: NOT ONLY ONCOLOGY

4.1 Hydrogels

Hydrogels are nets of polymeric materials embedding water molecules. When the net of monomeric chains is organized at nanoscale, they are considered nanomaterials (NMs). Nanogels show very selective properties, which lead to a variety of possible uses. Moreover, several of them are well tolerated when implanted in the CNS, or in direct contact with the brain.

Several hydrogels undergo sol–gel phase transition during the passage from the room to the inner body temperature (Figure 8). This property allows easy injection at the sol state, and rapid immobilization of the gel at the point of inoculation, that is the desired target. If loaded with antineoplastic drugs (irinotecan, camptothecin, vincristine, and paclitaxel), the release of the active principle was maintained for at least 28 days, with high levels and good antitumoral activity within a distance of 1–2 mm (Kim *et al.*, 2012; Ozeki *et al.*, 2012a,b; Ranganath *et al.*, 2009; Torres *et al.*, 2011). Further, functionalization with magnetic probes allowed the follow-up of long-term therapy (Kim *et al.*, 2012). A formulation of a thermosensitive and biodegradable hydrogel loaded with paclitaxel (OncoGel™) seemed to perform well in the rat against tumors growing in the CNS (Gok *et al.*, 2009; Tyler *et al.*, 2012; Vellimana *et al.*, 2013). A clinical trial for the treatment of brain tumors with this formulation was registered (NCT00479765), but terminated

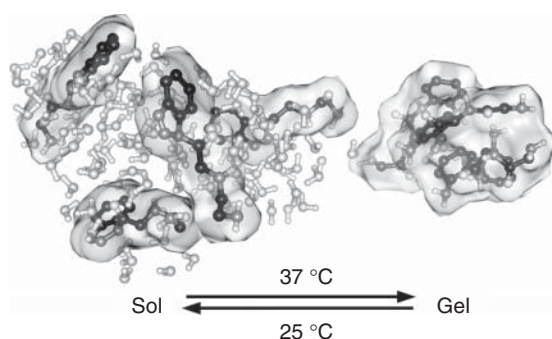


Figure 8. *In silico* simulation of the sol–gel transition of an hydrogel. [Obtained by Nanoengineer1 version 1-1-1]. Molecular graphics images and surfaces were produced by the UCSF chimera package from the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco, supported by NIH P41 RR001081 (Pettersen *et al.*, 2004).]

by sponsors before completion, after the failure of the OncoGel to fulfill the expectations for the treatment of esophageal cancer. A different use was developed by Nie *et al.* (2012), with a dye composed by a nanogel bound to the Coomassie blue and specifically targeted to the gliomas implanted in rats. They obtained a visible, blue delineation of the tumor that, in the opinion of the authors, could help a complete and selective surgical resection.

Hydrogels are not only useful for the treatment of tumors in the CNS, but they can also represent a suitable scaffold to induce controlled neurogenesis from precursors, as demonstrated *in vitro* (Table 4) and *in vivo* (Table 5). This is a fascinating aspect, suggesting the usefulness of these materials for repairing neurodegenerative or cavitory lesions of the CNS. To this end, Li *et al.* (2013) demonstrated that remyelinating oligodendrocytes developed from precursors and were able to repair the demyelination that was chemically induced in the nude rat. Alternatively, the implant of hydrogels into the hippocampal area in rats induced neuronal colonization, possibly useful to fill and repair cavity (Lim *et al.*, 2013). The composition of hydrogels implemented for CNS repair was analyzed by Medberry *et al.* (2013), which concluded that those derived from the CNS matrix seemed to perform better. Furthermore, functionalization with growth factors (Mohtaram, Montgomery, and Willerth, 2013, Song *et al.*, 2012) or erythropoietin (Wang *et al.*, 2012b) enhanced the regenerative properties. The property of other hydrogels, to rapidly increase their volume when

entering in contact with blood, suggested their exploitation for the treatment of intracranial hemorrhages or aneurysms. Hydrogel-coated coils were preferred to bare coils in selected cases, for the ease of positioning them and prolonged effect (Arthur *et al.*, 2005; Chao *et al.*, 2004; Gaba *et al.*, 2006; Sim, Shin, and Yoon, 2008). Because side effects have been reported (Dönmez *et al.*, 2009; Pickett *et al.*, 2007), the end point of studies registered at the ClinicalTrials.gov repository and reported in Table 6 is frequently the assessment of safety to efficacy ratio of these implant.

Thanks to their biocompatibility when in contact with the brain, hydrogels have also been tested in rats, to reduce the cerebral edema (McBride *et al.*, 2012), or as coats of cochlear implants, to improve the performance and prevent the degeneration of the auditory nerve (Chikar *et al.*, 2012). Dry, nanogel-based electrodes not requiring the direct scalp contact are under study to record continued registration of brain activity (Chi *et al.*, 2012; Foreman *et al.*, 2011).

4.2 Metallic Nanoparticles

The nanostructure of inorganic elements and molecules confers special features to the resulting nanomaterials. Iron oxide crystals whose size is lower than the size of ferromagnetic domain (ca. 30 nm) develop super paramagnetic properties widely exploited for potentiating imaging. Iron oxide NPs were classified by Cheng (2007) as ultra small paramagnetic iron oxides (USPIOs, 10–40 nm), monocrystalline iron oxide nanoparticle (MION, 10–30 nm), and cross-linked iron oxide (CLIO), which are MION covered by cross-linked dextran. In addition to the enhanced magnetic signal, they offer the advantage of a low uptake into the liver and the reticulum-endothelia. Approved for clinical use in humans for the MRI, they provide powerful enhancement of brain tumor imaging in comparison with larger particles, such as ferumoxides. However, their ability to target the tumor cells remains controversial. In fact, USPIO Ferumoxtran® accumulates in astrocytes and macrophages surrounding the tumor (Neuwelt *et al.*, 2004; Varallyay *et al.*, 2002). The i.v. injection of 10 nm USPIO [Ferumoxtran-10 (R)] in rats, engrafted with cells from human malignant glioma, enhanced the contrast of imaging proportional to the tumor growth and provided an

Table 4. Recent and selected experimental studies: *in vitro* performance of hydrogels as protective environment for neurons and glia, and their precursors.

System	Organ	Species	Effect	Hydrogel matrix	References
Tissue slices	Brain	Rat	Enhanced survival of explants	Agarose-based, indentation	Lee <i>et al.</i> (2011)
—	—	Mouse (embryo)	Axonal growth, neural migration	Matrigel ©	Gil and del Río (2012)
Mature neurons	Spinal cord	Rat	Neuronal mechanosensing	Cross-linked ssDNA	Jiang <i>et al.</i> (2010)
Mature neurons and glia	Hippocampus	Mouse	Enhanced survival	PEG-based, <i>F</i> : acetylcholine	Zhou <i>et al.</i> (2012)
—	—	Rat (embryo)	Mechanosensing of neurons by cocultured astrocytes	Polyacrylamide	Previtera <i>et al.</i> (2010)
Schwann cells	Nerve	Neonatal rat	Proliferation, NGF and BDNF secretions, polarization of cocultured neurons	3D, collagen and HA	Suri and Schmidt (2010)
NSPCs	—	Mouse	Enhanced survival to stress	Collagen, HA, HpS	Zhong <i>et al.</i> (2010)
—	—	—	Differentiation (GABAergic neurons)	Gelatine and alginate acid <i>F</i> : RA	Addae <i>et al.</i> (2012)
—	—	Embryo rat	2D: increased glia	2D and 3D	Lampe <i>et al.</i> (2010)
—	—	Adult rat	3D: proliferation, mechanosensing	PEG-based	—
—	—	—	Neuronal differentiation	MAC; <i>F</i> : IFN- γ , BDNF, EP	Leipzig <i>et al.</i> (2010)
NSPCs and astrocytes	CNS	Neonatal rat	Differentiation, mechanosensing	HA	Seidlits <i>et al.</i> (2010)
Primary neurons	Brain	Chick embryos	Adhesion, differentiation, mechanosensing	Dextran and chitosan in methylcellulose and agarose	Zuidema <i>et al.</i> (2011)
Neural stem cells	—	Rat	Proliferation	Collagen; <i>F</i> : EGF	Egawa <i>et al.</i> (2011)
—	—	Human	Migration	Multi-arm thiolated PEG; <i>F</i> : HGF, LIF	Li <i>et al.</i> (2012)
—	—	Human	Survival in stress, proliferation, differentiation	Gtn-HPA	Lim <i>et al.</i> (2012)
Stem cells	Hippocampus, spinal cord,	Human	Biocompatibility; differentiation (glia and neurons).	Dextran/gelatin and laminin	Jurga <i>et al.</i> (2011)
Stem cells and pericytes	Umbilical vein endothelium, Brain	Human	Collagen IV and laminin deposition	PEG diacrylate; <i>F</i> : ephrin A1	Saik <i>et al.</i> (2011)
PC12	—	Rat	Adhesion, neurite extension	IPN hydrogel films	Lu <i>et al.</i> (2012a)
MGB cells	—	—	Mechanosensing, RGD chemotaxis	HA, <i>F</i> : RGD	Ananthanarayanan, Kim, and Kumar, (2011)

B27, neurotrophic factor; BDNF, brain-derived neurotrophic factor; CNS, central nervous system; ssDNA, single-stranded deoxyribonucleic acid; EGF, epithelial growth factor; EP, erythropoietin; *F*, functionalization; GABA, gamma-aminobutyric acid; Gtn-HPA, gelatin-hydroxyphenylpropionic acid; HA, hyaluronic acid; HGF, hepatocyte growth factor; HpS, heparin sulfate; IFN- γ , interferon- γ ; IPN, interpenetrating polymer networks; LIF, leukemia inhibitory factor; MAC, methacrylamide chitosan; NGF, neural growth factor; NSPCs, neural stem precursor cells; PC12, pheochromocytoma cells; PEG, polyethylene glycol; RA, retinoid acid and RGD, tripeptide.

Table 5. Recent and selected experimental studies: *in vivo* performance of hydrogels as protective environment for neurons and glia, and their precursors.

Species	Result(s)	Hydrogel matrix	Functionalization	References
Rat	Nigro-striatal neuronal regeneration	PEG-based hydrogel	BDNF and GDNF	Lampe <i>et al.</i> (2011)
Rat	Neural colonization, chemotaxis	Gtn-HPA	Polymeric NPs with chemotactic factors	Lim <i>et al.</i> (2013)
Rat	Microglia and astrocytes do not colonize	TGP (xyloglucan)	poly-D-lysine	Nisbet <i>et al.</i> (2010)
Rat	Survival and differentiation (olfactory glia)	Gellan gum	GRGDS	Silva <i>et al.</i> (2012)
Rat	Survival and axon growth (neurons)	PiPAA and PEG or MCELL	BDNF (released)	Conova <i>et al.</i> (2011)
Rat	Neuronal proliferation without glial reaction	DX/gelatin and laminin	—	Jurga <i>et al.</i> (2011)
Nude rat	Oligodendrocyte proliferation, remyelination	HA and gelatin	Thiol; RGD	Li <i>et al.</i> (2013)
Rat, spinal cord injuries	Minimal formation of scars, no inflammatory astrocytes	HA	—	Khaing <i>et al.</i> (2011)
Rat, spinal cord injuries	Stem cells survive and proliferate, but results with BDNF	HA	MMP; IKVAV; BDNF	Park <i>et al.</i> (2010)
Rat, stroke	Reduced volume of the lesion	Alginate	VEGF	Emerich <i>et al.</i> (2010b)
Rat, stroke	Neuronal precursors survive and differentiate	HA and HpS	—	Zhong <i>et al.</i> (2010)
Rat model, HD	Striatal neurons survive the excitotoxic damage	Alginate	VEGF	Emerich <i>et al.</i> (2010a)
Rat, tumor C6	Prolonged release of drugs, post-tumoral resection	Drug/PLGA/TGP	CPT, VRCT	Ozeki <i>et al.</i> (2012a,b)
Mice, stroke	Survival and colonization by neuronal precursors	HA/MCELL	Erythropoietin	Wang <i>et al.</i> (2012b)
Mice, stroke	Transplant and differentiation of stem cells	TGP	—	Osanai <i>et al.</i> (2010)
Guinea pig	Enhanced signal from implanted electrodes (auditory cortex) coated with the hydrogel	Alginate	Conducting polymer ^a	Kim <i>et al.</i> (2010)
Rat	Enhanced light-evoked action potentials (hippocampal implants)	Conducting hydrogel	—	Lu <i>et al.</i> (2012a)

BDNF, brain-derived neurotrophic factor; C6, experimental malignant glioma; CPT, camptothecin; DX, dextran; GRGDS, fibronectin-derived synthetic pentapeptide; GDNF, glial-derived neurotrophic factor; Gtn-HPA, gelatin-hydroxyphenylpropionic acid; HA, hyaluronic acid; HD, Huntington's disease; HpS, heparin sulfate; IKVAV, esapeptide from laminin; MCELL, methylcellulose; MMPs, matrix metalloproteinases peptides; NPs, nanoparticles; PC12, pheochromocytoma cells; PEG, polyethylene glycol; PiPAA, polyisopropylacrylamide; PLGA, poly(lactic-co-glycolic acid (microspheres)); RGD, tripeptide; TGP, thermoreversible gelation polymer; VCR, vincristine; VEGF, vascular endothelial growth factor.

^aethylenedioxythiophene.

indirect measure of tumor proliferation (Kremer *et al.*, 2007). In a model of multiple sclerosis, USPIO provided a more powerful imaging than gadolinium, with an evident signal when no other signs of the disease were detectable (Millward *et al.*, 2013).

Silica nanospheres (124 nm, Figure 7d) loaded with USPIO accumulated in the brain, and their main site of excretion was through liver and spleen. It was possible to improve the amount of NPs in

the brain by applying an external magnetic field. In this case, the NPs were able to cross the BBB, and accumulated also in the parenchyma. The crossing of the BBB seemed to proceed through internalization of NPs into the endothelial cells, as demonstrated *in vitro*. The silica magnetic NPs could also carry drugs, in this case an anti-amyloid agent, and release them on demand, when a magnetic field was externally applied and switched off (Kong *et al.*, 2012). To better address the magnetic NPs to the

Table 6. Clinical studies implementing commercial hydrogel preparations for intracranial hemorrhage, brain traumas, and dural incision registered at the ClinTrials.gov repository.

NCT	Start date	End date	Disease	Administration	Formulation	Type and phase	Country
01516658	February 2012	January 2014	IA, SH	Implant	Hydrogel coil versus bare Pt coil	Interventional, IV	Japan
01407952	April 2012	October 2018	IA	Implant	HydroCoil versus bare Pt coil	Interventional	USA
00626912	June 2007	June 2013	IA, SH	Implant	Hydrogel coil versus bare Pt coil	Interventional, IV	Canada
01195129	December 2008	December 2013	CA	Implant	HydroCoil (MicroVention) versus non-hydrogel coils	Interventional, IV	USA
00890604	July 2009	July 2010	SH; TBJ, severe	Topical	Cooling pads	Interventional, ?	USA
01354509	May 2011	May 2013	TBI	Topical	Cooling pads	Interventional, ?	USA
00704340 ^a	Sep 2005	May 2009	Dural incision	—	Sealant, DuraSeal®	Interventional, IV	USA

CA, cerebral aneurysm; IA, intracranial aneurysm; SH, subarachnoid hemorrhage; and TBJ, traumatic brain injury.

^aOne study had published results (Osun *et al.*, 2012).

neural parenchyma, the surface functionalization by molecules supposed to facilitate the crossing of the BBB could be of help. When administered intravenously to rodents, these NPs crossed the BBB without damaging the tight junction, and easily entered the three main components of the brain parenchyma, endothelia, astrocytes, and neurons. *In vitro* studies showed that they accumulated into the cytoplasm (Yim *et al.*, 2012). PEG-coated USPIO were protected from too rapid clearance inside the reticulo-endothelia and the functionalization with lactoferrin enhanced their uptake into the brain endothelia *in vitro*. After i.v. injection in rats, these NPs reached high concentration in the parenchyma of the brain. The *in vivo* pharmacodynamic suggested that the crossing process involved the specific binding to lactoferrin receptors (Qiao *et al.*, 2012).

Ceria NPs (7–9 nm), after brain perfusion in rats, did not damage the BBB and accumulated in the choroid plexus at higher concentrations than in other eight different regions of the brain (Dan *et al.*, 2012).

Maintaining the integrity of tight junctions is important to avoid vascular damage to the brain. Some metallic NPs have been shown to induce such damage, as demonstrated for alumina and TiO₂ NPs. Both NPs suppressed the markers of tight junctions, claudin-5, and occludin (Chen, Zhang, and Toborek, 2013; Brun, Carrière, and Mabondzo, 2012). These NPs are therefore to be considered toxic for the CNS.

5 THE NANOMEDICINE FOR THE POSTERIOR POLE OF THE EYE

The posterior pole of the eye is affected by diseases leading to severe visual impairment and irreversible blindness. In addition to tumoral growth, the other relevant event in this site is the retinal and choroidal neovascularization. It is present in nearly all pathologies, included the most common ones, such as diabetic retinopathy, malignant hypertension, age-related macular degeneration, and choroidal infections and inflammation (Campochiaro, 2000).

The only antineoplastic drug developed for local implant is the Marquibo®, for metastatic, malignant uveal melanoma (ClinicalTrials.gov, NCT00506142). To control neovascularization, the most widely used NMs are implants of nanoporous silicon or biodegradable polymeric shells, which release steroid antiinflammatory or antiviral agents, to treat age-related macular degeneration and the choroiditis (Edelhauser *et al.*, 2010). The nanoporous silicon is a nanostructured material whose size largely exceeds the nanoscale. Its uniform porosity confers high stability to the implant (Cheng *et al.*, 2008), which can release steroid antiinflammatory drugs for some years. The small size and the high biocompatibility of the material reduce to a minimum the need of invasive procedures and risks following repeated penetration of the eye. However, the prolonged (1–2 years) delivery of high doses of steroids caused a prevalence of long-term

side effects as high as 40% for cataract and 100% for chronic glaucoma. The implants releasing lower doses of steroids were better tolerated on the long term (Edelhauser *et al.*, 2010). A different and investigative approach to the clinical treatment of age-related macular degeneration consists of polymeric shells containing stem cells releasing the ciliary neurotrophic factor (Kauper *et al.*, 2012).

In contrast with the limited clinical exploitation, the experimental studies *in vitro* and *in vivo* open the field to a wider range of materials, drugs, and innovative solutions. As mentioned earlier, angiogenesis is relevant for further anatomical and functional degenerations of the tissues at the posterior pole. Several factors and drugs regulate these events, triggering inductive or aborting pathways, and nanotechnology can focus on them. Silver NPs, dendrimers, immunoliposomes, and polymeric NPs inhibited angiogenetic factors, or released anti-angiogenetic ones (Kalishwaralal *et al.*, 2010; Li *et al.*, 2010; Marano *et al.*, 2005; Park *et al.*, 2009), without toxicity for the normal cells of the retina. Drugs active against inflammation and neovascularization, such as minocycline, tamoxifen, and steroids, were released to the posterior pole by nanoliposomes (Kaiser *et al.*, 2012) or polymeric NPs, eventually entrapped in thermosensitive hydrogels (Boddu *et al.*, 2010; De Kozak *et al.*, 2004). Polymeric NPs provided excellent bioavailability of steroids at the posterior pole also after i.v. injection in a model of experimental autoimmune choroiditis in rats. The NPs appeared in the retina and choroid after 3 h, and were still present there after 7 days. The clinical and histological signs of inflammation were reduced within 24 h and ameliorated during the following week without toxic reactions (Sakai *et al.*, 2006). The surface functionalization with the peptide cyclo-(Arg-Gly-Asp-D-Phe-Cys) addressed NPs to the chorionic and retinal endothelia, through selective receptor binding (Pollinger *et al.*, 2013).

Anionic albumin NPs were tested as carriers of ganciclovir, for the treatment of viral chorioretinitis and uveitis. They diffused across the 3D net of collagen and hyaluronic acid characterizing the vitreous, reached the posterior pole, and there sustained the release of the drug for at least 2 weeks. Studies in rats did not show signs of inflammation, degeneration, or autoimmunity in the retina or in the choroid (Kim, Robinson, and Csaky, 2009; Merodio *et al.*, 2002). Lipid NPs and compacted DNA NPs promoted the

transfection of gene and were proposed for the treatment of selected retinal diseases, for which gene therapy could be appropriate (Del Pozo-Rodríguez *et al.*, 2008, 2013; Cai *et al.*, 2009). Also chitosan and, more safely, magnetic NPs promoted transfection of the retina (Prow *et al.*, 2008). Chitosan NPs were better tolerated if the surface is decorated with the STY tripeptide (Jayaraman *et al.*, 2012).

Fullerols, Au, TiO₂, and ZnO induced retinal damage, mainly through phototoxicity, and should therefore be regarded as toxic (Kim *et al.*, 2013; Sanders *et al.*, 2012; Wielgus *et al.*, 2010; Gao *et al.*, 2013). Others NMs can just quench the oxidative stress, such as NPs of cerium oxide (Chen *et al.*, 2006). Recently, Wong *et al.* (2013) demonstrated high and prolonged (up to 120 days) bioavailability of nanoceria in the retina after intravitreal injection, without toxic effects.

An approach to retinal diseases, which seems futuristic, but also promising *in vitro*, implements nanotechnologies to provide scaffolds for retinal regrowth from stem cell and for retinal prosthesis. Films of organic polymers of polycaprolactone decorated with nanolithography promoted the encapsulation, replication, differentiation, and release of retinal cell precursors obtained from stem cells (Sodha *et al.*, 2011). Similarly, coating with NPs the surface of implants for retinal prosthesis enhanced the surface roughness that, in turn, stimulated the proliferation of precursor stem cells. According to Maul *et al.* (2013), this method could eventually evolve into a treatment of the blindness caused by the degeneration of the pigmented epithelia, when the neuronal cells and ganglia are preserved.

The development of adequate nanotechnologies could improve not only treatment but also diagnosis. Quantum dots (QDs), when bound with antibodies against a marker for astrocytes and Müller cells, provided unequivocal imaging of gliosis, a poorly known aspect of retinal damage. QDs suspended in aqueous solutions clearly delineated the vitreous extensions, and gave a visible trace for easier surgical manipulation (Yamamoto *et al.*, 2007).

6 CONCLUSIONS

Nanotechnology has been able to stimulate the doldrums of a field, the treatment of disease in the CNS and in the posterior pole of the eye, which

seemed definitely limited by the presence of selective biological barriers. Inventions, inconceivable until few decades ago, included not only carriers for drugs and smart probes for imaging but also new models for toxicity studies and phantoms capable to mimic the properties of the brain matrix. Even an apparently steadfast cornerstone of neurology, the inability of nervous tissue to regenerate, has fallen after the production of nanostructured shells in which precursors can find a favorable environment for reproducing and differentiate into mature neurons and specialized glia. Experimental *in vivo* evidences suggested the possibility that these scaffolds could be used to repair demyelination, regenerate tissue retaining nervous functions, and refill cavitory lesions in the CNS.

This exciting context has been confirmed by translational medicine. The exploitation of nanotechnology in the neurology and ocular fields has in common improved efficiency and safety, minimal need for invasive procedures, and precise modulation of drug release. In particular, the finely tunable properties of hydrogels seem to be exceptionally appropriate. Therefore, NMs seem to approximate the ideal means of treatment, which should be safe, biodegradable without toxic residuals, efficacious, and manageable. Many NMs are designed to act as theranostic agents in the real term, providing means to target the lesion, define, and treat it. The therapeutic effect could be obtained on-demand by modifying the environment, and the biocompatibility of the NMs permits their persistence at the site of action for long periods, even for years, warranting long-term follow-up and deferred intervention on the lesion. Nanodrugs can be advantageous even for the diseases charged with the most severe prognosis, the malignant glioblastoma and the metastatic tumor to the brain.

The only gray area is the medium to long-term toxicity of NMs and their by-products. Up to now, long-term side effects have been described after 3 years in patients implanted with nanoporous devices in the vitreous chamber. The toxicity seemed however to be due to the prolonged and too sustained release of steroids, not to the NM itself.

Our conclusions are that the nanomedicine applied to the CNS and the eye represents an important improvement of diagnosis and treatment of poorly accessible lesions. However, the accumulation of NMs or of products of dissolution remains to be carefully evaluated as a possible source of

long-term and poorly predictable toxicity. Before further extending the exploitation of this technology, these aspects should be known as well as possible, with special attention to the low clearance in these anatomical sites. A diligent, but rational approach should be encouraged, to avoid both delay in therapeutic advancement and additional hazard because of careless behavior.

RELATED ARTICLES

Chapter 6

Chapter 14

Chapter 17

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FURTHER READING

ClinicalTrials.gov <http://www.clinicaltrials.gov/>

Part Three

Stem Cell Toxicology

Stem Cells in Toxicity Testing

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1 INTRODUCTION

In “Toxicity Testing in the 21st Century: A Vision and a Strategy,” the National Research Council advocated the use of *in vitro* systems and pathway analysis for detecting toxicants (National Research Council (NRC), 2007). A number of publications have since suggested that stem cells, and in particular, human stem cells, be used for some of these tests. The use of human cells would eliminate the extrapolation from nonhuman or nonmammalian tests. However, there are a number of concerns involving the use of human stem cells. First, if human embryonic stem cells (hESCs) are to be used, there are legal and ethical issues with which to contend. Second, differentiating stem cells do not differentiate along the same pathway; that is, within a culture of differentiating stem cells, there is often a variety of differentiated cell types complicating analysis. For example, in the mouse embryonic stem cell test (mEST), it has been estimated that only 17–25% of cells in the total population had differentiated to cardiomyocytes, the desired cell type, after 7 days of culture (Seiler *et al.*, 2004).

Human induced pluripotent stem cells (iPSCs) alleviate some of the legal and ethical issues surrounding the use of hESCs. iPSCs are adult somatic cells that have been reprogrammed by the addition of various signaling molecules. These reprogrammed cells then perform more like hESCs than like the adult somatic cells from which they were derived. iPSCs have a number of other advantages as well. These include the ability to use any somatic cell type, the ability to use cells with a

variety of genotypes, the ability to test for efficacy or toxicity to the individual from whom the cells were derived, and finally the cells are autologous (see Chapter 16). However, studies have demonstrated that they may differ from hESCs in gene expression and other characteristics [reviewed by Wobus and Löser (2011)].

Nonetheless, stem cells are being used for toxicity testing in some specific cases. The situation in which stem cells have the greatest applicability may be developmental toxicity testing. Additionally, systems for developmental neurotoxicity testing are increasingly being developed. These areas are discussed later in more detail.

2 DEVELOPMENTAL TOXICOLOGY

2.1 The Validated Test

ECVAM (European Centre for Validation of Alternative Methods) was established by the European Union in 1993 and sponsored a workshop 2 years later to discuss *in vitro* screening methods for reproductive and developmental toxicity (Brown *et al.*, 1995). A recommendation from this workshop was to validate and improve existing tests as well as to develop new tests. Three *in vitro* tests were selected for validation; the mEST was the only one of the three that did not include living animals using only two commercially available cell lines. The D3 mouse embryonic stem cell (mESC) line from the 129 mouse strain (Doetschman *et al.*, 1985) is used as the embryonic stem cell line and the 3T3

lung fibroblast line is used to model a differentiated cell. The mEST was first described by Spielmann *et al.* (1997) and tests the differentiation capacity of the D3 cell line. Differentiation is initiated through the formation of embryoid bodies (EBs), which are aggregates of cells that resemble the inner cell mass of early embryos. EBs have the ability to differentiate into cells of endodermal, mesodermal, and ectodermal origin; EBs are formed in “hanging drops” by suspending cells in culture media from the lid of petri dishes. After 3 days, the EBs are transferred to suspension culture, where they continue to grow and differentiate for an additional 2 days. Individual EBs are then seeded into the wells of a 24-well culture dish and are assessed for contracting cardiomyocytes on day 10 of culture. A range of test article concentrations is added to the culture media for the entire culture period starting at the time of EB formation, although more recent modifications of the test system include the test articles beginning on day 3 of culture to attempt to separate effects on proliferation from effects on differentiation (van Dartel *et al.*, 2009b). On day 10, the percentage of culture wells containing contracting cardiomyocytes is determined and compared to the solvent control wells from which an ID_{50} (concentration of test compound inhibiting cardiomyocyte differentiation by 50%) is calculated. Additionally, cytotoxicity is determined in the 3T3 fibroblast line, to mimic the effect on maternal tissue/organs, as well as in undifferentiated D3 mESCs, to represent embryo-like effects. A prediction model is then used to classify the compounds into one of the three categories—nonembryotoxic, weakly embryotoxic, or strongly embryotoxic.

The ECVAM validation tests showed that the mEST correctly categorized 78% of the 20 selected test chemicals; additionally, the assay correctly predicted the six strong embryotoxicants included within the set of test chemicals (Genschow *et al.*, 2002). In 2008, the mEST was accepted by ECVAM as a validated *in vitro* assay to screen for developmental toxicity (Spielmann *et al.*, 2008). Currently, the mEST is being used in some pharmaceutical and chemical companies to aid in prioritizing compounds undergoing development and to screen chemicals early during the product development pipeline to reduce the late attrition rate (Marx-Stoelting *et al.*, 2009).

2.2 Additional Compounds Screened in the mEST

However, reports have suggested that the mEST may not be as predictive with some compounds, especially if those compounds are highly cytotoxic (Chapin *et al.*, 2007; Marx-Stoelting *et al.*, 2009). When testing 19 in-house pharmaceutical compounds with known *in vivo* developmental toxicity activity, Paquette *et al.* (2008) found that a modified mEST correctly identified only 53% of these new compounds even though their results with the ECVAM validation compounds were nearly identical to the ECVAM results. Addition of several approved pharmaceutical compounds with low or moderate risk increased the predictivity of the mEST to 85%. The authors pointed out several concerns with the test such as compounds with low solubility; the low solubility inhibited the ability to determine cytotoxicity and limited the predictivity of the test. They also pointed out that the prediction model may need to be changed because the low-risk compounds used in its development were not very cytotoxic, while many of the low- and moderate-risk compounds tested by Paquette *et al.* (2008) demonstrated cytotoxicity in the differentiated 3T3 cells at lower concentrations than either the cytotoxicity induced in the mESCs or the inhibition of differentiation. In another study, Chen *et al.* (2010) tested six compounds used in cosmetics; in general, the prediction from the mEST was in agreement with data from whole animal studies.

Okadaic acid, a marine toxin produced by dinoflagellates, was observed to inhibit differentiation of mESCs into beating cardiomyocytes (Ehlers *et al.*, 2010). The authors applied the ECVAM prediction model to their results claiming that the compound was weakly embryotoxic. This claim of weak embryotoxicity appears to have been derived primarily from the fact that the ID_{50} was slightly lower than the IC_{50} for D3 cells, both of which were slightly lower than the IC_{50} for 3T3 cells. Okadaic acid was embryotoxic in a different assay, which examined differentiation of teratocarcinoma F9 cells carrying a reporter gene. Additionally, the compound had been tested in the frog embryonic teratogenicity assay—*Xenopus* (FETAX) and was observed to produce delayed growth and malformations at more than a 10-fold higher concentration than the ID_{50} in the mEST.

A problematic compound is fluoxetine. This member of the selective serotonin reuptake inhibitor class has been reported to result in poor neonatal adaptation as evidenced by several behavioral features such as jitteriness, poor tone, weak or absent cry, and diminished pain reactivity (Hines *et al.*, 2004). However, data on *in vivo* developmental toxicity in animal models are equivocal with decreased birth weight and survivability observed in rats (Vorhees *et al.*, 1994) or no adverse effects observed in guideline rat and rabbit studies (Byrd and Markham, 1994). Examining this compound in the mEST, Kusakawa *et al.* (2008) reported that the compound was strongly embryotoxic, which is not in agreement with the animal data from the guideline-type studies by Byrd and Markham (1994). Furthermore, Kusakawa *et al.* (2008) examined expression of markers of the undifferentiated state, endoderm derivatives and mesoderm derivatives; they found that fluoxetine tended to enhance expression of endodermal and mesodermal markers early in the culture, but the undifferentiated markers were enhanced later in the culture period. They interpreted these observations to support recent data suggesting that fluoxetine may produce cardiac defects by potentially inhibiting mesodermal differentiation.

The mEST has also been used to evaluate the relative potency of closely related compounds. de Jong *et al.* (2009) tested four metabolites of glycol ethers as well as two parent compounds and two structural analogs. Metabolites of the glycol ethers are considered to be the proximate developmental toxicants, and the results from the mEST demonstrated that the metabolites inhibited differentiation into beating cardiomyocytes while the two parent compounds had no effect on differentiation. Additionally, the metabolites showed the same relative potency in the mEST with *in vivo* data. de Jong *et al.* (2011b) also compared the embryotoxicity of valproic acid and five structurally related analogues that differed in their *in vivo* embryotoxic activity. When comparing the potency to induce exencephaly or to inhibit cardiomyocyte differentiation, there was good correlation between the two endpoints, suggesting that the mEST is capable of determining the relative potency of a group of similar compounds. The authors attempted to make the assay more quantitative by monitoring expression of two cardiac markers using flow cytometry. Despite a good deal of variability in the gene expression data,

the same relative order of potency of the analogs was achieved at the ID₅₀ concentration. The same analogs and one additional analog were tested by Riebeling *et al.* (2011) using the validated mEST and a modified assay that used flow cytometry of intracellular proteins as the endpoint rather than beating cardiomyocytes [FACS-EST, (fluorescence activated cell sorting–embryonic stem cell test) as described below]. There was good agreement between the *in vivo* teratogenic activity of all seven compounds and the results with both of the mEST protocols. Overall, these results suggest that the mEST is capable of differentiating between closely related compounds that differ in their developmental toxicity potential.

Several publications have used the mEST to screen chemicals for developmental toxicity. Park *et al.* (2009) used silica nanoparticles of various sizes in the mEST and observed that the supposedly smaller nanoparticles (10 and 30 nm) inhibited differentiation of the mESTs but there was no adverse effect of the larger nanoparticles (80 and 400 nm). However, when the nanoparticles were characterized, it was found that the particle sizes specified by the company to be 30, 80, and 400 nm were incorrect. The 400-nm particles were approximately 248 nm, while the 30- and 80-nm particles were both about 34 nm. Part of the difference in response of the mESTs to the particles of approximately the same size may have been due to differences in the zeta potential. Dispersion of nanoparticles with a zeta potential in the range of the 80-nm nanoparticles is less stable; dynamic light scattering results indicated differences in the dispersion of the 30- and 80-nm nanoparticles, suggesting that zeta potential may have had effects on dispersion of similar sized nanoparticles. Park *et al.* (2009) could not correlate their *in vitro* results to animal studies, because no *in vivo* results were available for these particles. However, their results suggested that the nanoparticles might be embryotoxic *in vivo*. Di Guglielmo *et al.* (2010) examined gold and cobalt ferrite nanoparticles for embryotoxicity. They found that the addition of the nanoparticles interfered with normal EB formation, so they only exposed the cells to the nanoparticles during the EB phase, or the first 5 days of culture. The EBs were then plated in normal control media for the final 5 days of culture. The authors observed that the nanoparticles were less cytotoxic and embryotoxic than their corresponding salts primarily because of

the coatings that were applied, such as hyaluronan, aminosilanes, or thiol-silanes. Similar to Park *et al.* (2009), these authors could not relate their *in vitro* results because no *in vivo* results were available for these particles. They concluded that the nanoparticles that they tested were predicted to only be weakly embryotoxic or nonembryotoxic in the case of cobalt ferrite nanoparticles coated with gold.

3 SUGGESTED MODIFICATIONS TO THE MEST

3.1 Automating Cardiomyocyte Analysis

A number of modifications have been suggested for the mEST [reviewed by Buesen *et al.*, 2004, and Marx-Stoelting *et al.*, 2009]. Several of the suggested modifications deal with the endpoint of beating cardiomyocytes. When only 25% or less of the cells differentiate to beating cardiomyocytes (zur Nieden *et al.*, 2001), it can be difficult to visualize this microscopically. Additionally, counting the number of wells with beating cardiomyocytes is not a very quantitative approach. Automating image analysis was used by Peters *et al.* (2008), who found no significant differences between manual and automated counting with four chemicals from the ECVAM validation study. However, they did report a great deal of variability with the automated counting due to the 3D structure of the EBs. Koseki *et al.* (2010a) used field potential detection as a method to detect beating cardiomyocytes. EBs were seeded into gelatin-coated wells with a microelectrode array present and allowed to differentiate for 5 days. The authors examined the wells microscopically for beating cells as well as detected the field potential using a microelectrode array system. There was approximately 90% correlation between these two endpoints. When testing several of the compounds from the ECVAM validation study, the authors found that they were able to correctly predict the class of embryotoxicity for all of the chemicals using either microscopic evaluation or microelectrode array detection. They also included expression of cardiac troponin protein to further test the ability of the microelectrode array assay to differentiate between beating cardiomyocytes and skeletal muscle cells (Koseki *et al.*, 2010b). They found that the area showing field potential also stained for cardiac troponin protein, suggesting

the cells creating the field potential were cardiomyocytes; furthermore, they demonstrated that the embryotoxicant compound 5-fluorouracil decreased the field potential and cardiac troponin expression. Recently, impedance monitoring using the xCELLigence real-time cell analyzer cardio system has been applied to human and mouse stem cells which were differentiated to cardiomyocytes (Jonsson, Wang, and Becker, 2011). Although the focus of that study was not the mEST, this method may be useful in making the scoring of beating cardiomyocytes more automated and quantifiable.

3.2 Adding Metabolic Capability

A major criticism of nearly all *in vitro* tests concerns the lack of metabolism. One way to address this is to separately test known metabolites of a compound. This approach was used by Eckardt *et al.* (2012) in testing the antihelminthic drug albendazole and its major metabolite, albendazole sulfoxide. Various concentrations of each of these compounds were tested using the validated mEST assay; the authors also tested combinations of the two compounds (80% parent : 20% metabolite and 50% parent : 50% metabolite) to simulate incomplete metabolism of the parent compound. The authors found that the parent compound inhibited differentiation at a concentration that was approximately 40 times lower than the ID₅₀ concentration of the metabolite; both mixtures inhibited differentiation at concentrations very similar to that of the parent compound. As they were focusing only on development and were comparing two different *in vitro* assays, they omitted the cytotoxicity evaluation in 3T3 cells; therefore, they could not predict whether the parent compound would be classified as weakly or strongly embryotoxic. Another method to include metabolic activation was suggested by Hettwer *et al.* (2010). These authors added either cyclophosphamide or valpromide to cultured hepatocytes; the supernatant containing presumed metabolites was included in culture medium for the mEST. When cyclophosphamide was incubated with mouse hepatocytes and then used in the mEST, there was a greater inhibition of differentiation than with nonincubated cyclophosphamide. No difference was observed between incubated and nonincubated valpromide; when analyzed by GC/MS (gas chromatography–mass spectrometry), the major

metabolite of valpromide, valproic acid, was not produced by mouse hepatocytes. Furthermore, the authors found that valproic acid was produced if valpromide was incubated with human hepatocytes, but this mixture was not tested in the mEST.

3.3 Monitoring Gene Expression

Many of the attempts to make the assay more quantitative have involved evaluation of gene expression. zur Nieden *et al.* (2001) were among the first to include molecular markers. Using semiquantitative PCR (polymerase chain reaction), they examined expression of α - and β -myosin heavy chain genes; they were able to correctly categorize the seven ECVAM validation study chemicals tested when substituting gene expression for the beating cardiomyocyte portion of the assay. A stem-cell-based reporter assay was developed by Uibel *et al.* (2010) and termed the *ReProGlo assay*. Undifferentiated mESCs were transfected with a luciferase reporter gene under the control of a *Wnt* promoter; significant increases or decreases in reporter activity were monitored. The *Wnt*-signaling pathway was chosen as it plays an important role in early embryonic development and is highly evolutionarily conserved. Seventeen chemicals were tested; three of these chemicals had no teratogenic activity and had no effect on reporter expression. In total, 10 of the 14 embryotoxic compounds had significant effects on *Wnt* expression. The authors concluded that the assay had a good deal of promise as the treatment time was only 24 h; there was little cytotoxicity produced during this short exposure time and the assay could be conducted in 96-well plates decreasing the cost. Suzuki and colleagues used a similar approach by modifying the mEST to quantify *Hand1* or *Cmya1* expression as markers of differentiation. They had previously identified these two genes as well as several others whose expression was increased during differentiation into cardiomyocytes and was decreased by treatment with embryotoxicants (Suzuki *et al.*, 2011a). They developed stable transgenic cell lines that had either the *Hand1* or the *Cmya1* promoters upstream of a luciferase reporter gene (Suzuki *et al.*, 2011b). They tested a total of 24 chemicals using both cell lines; the chemicals were categorized as nonembryotoxic, strongly embryotoxic, or weakly embryotoxic. The authors classified the 24 chemicals using the

ECVAM prediction model but replaced the differentiation assay with gene expression data. The overall accuracy of the EST conducted with the *Hand1* transgenic line was 83% and 92% with the *Cmya1* transgenic line; these compared very favorably with the 81% for the validated mEST (recalculated based on the 24 chemicals used in this study). This method offered several advantages including a shorter culture period (6 days), use of smaller amounts of culture media and cells because of the use of 96-well plates, less labor-intensive culture conditions because of the elimination of the EB step, and a higher throughput endpoint in chemiluminescent detection of differentiation. The authors then conducted a cross-laboratory prevalidation study using their modified assay tested across six compounds of the three classes used in the ECVAM validation in four different laboratories (Suzuki *et al.*, 2012). They found that the experimental protocol transferred well with an overall prediction accuracy of 79% for both modified assays for the six chemicals in the four laboratories. Two chemicals, hydroxyurea and ascorbic acid, were misclassified in both modified test systems; the misclassification was by one category. Hydroxyurea, a strong embryotoxicant, was classified as weakly embryotoxic in three of the four laboratories in both assays, while ascorbic acid, a nonembryotoxicant, was classified as weakly embryotoxic in two of the four laboratories in both assays.

Global gene expression has also been used as an endpoint in the mEST (reviewed by van Dartel and Piersma, 2011). EBs are made up of a variety of cell types, each expressing different genes. van Dartel *et al.* (2009a) were among the first to apply global gene expression to the mEST when evaluating the embryotoxicity of monobutyl phthalate. They observed significant changes in 48 genes after only 24 h of treatment; as many of these genes were included in the “development,” “embryonic development,” or “morphogenesis” categories using gene ontology, they reanalyzed their data using gene set enrichment analysis. This reanalysis demonstrated no significant changes in individual genes with monobutyl phthalate treatment but did demonstrate effects on sets of genes. They extended this work by following changes in individual genes from control EBs over time; they termed these changes as the *differentiation track* (van Dartel *et al.*, 2010a). When EBs were treated with monobutyl phthalate or 6-aminonicotinamide for up to 96 h, the

authors could detect deviation from the control differentiation track within 24 h. The authors altered the culture system and examined gene expression only for the first 48 h of culture (van Dartel *et al.*, 2010b); they examined gene expression of control and EBs treated with a single concentration of each of five different developmental toxicants or the nontoxicant, penicillin G. They defined a different differentiation track that consisted of only 26 genes that were altered with time in the controls and responded to treatment with the toxicants. Using a leave-one-out cross-validation analysis of the data on the six compounds and the controls, they found correct significant deviations in 27 of 30 cases. The authors concluded that this assay showed promise to detect developmental toxicants as well as supplying some preliminary information on possible mode of action of the compounds.

The authors extended this work by examining 12 compounds, most of which were not included in the validated mEST (van Dartel *et al.* 2011a). They compared the gene expression profiles of a single concentration of these 12 chemicals to the differentiation tracks that they had previously described (van Dartel *et al.*, 2009a, 2010a). The overall accuracy of predicting either a positive or a negative embryotoxic effect was 83% with the differentiation track defined by van Dartel *et al.* (2009a) and was 67% for the track defined by van Dartel *et al.* (2010a). While the assay generally correctly predicted developmental toxicants, it was less robust with nontoxicants. An explanation for this effect may involve, in a large part, concentration. Owing to the expense of the assay, only a single concentration of each chemical was utilized; the addition of concentration-dependent information may aid in correctly predicting the developmental toxicity potential of these compounds. The authors found that the ability to predict developmental toxicity in this assay was dependent on the concentration when they tested various concentrations of flusilazole (van Dartel *et al.*, 2011b).

The pairing of transcriptomics and the mEST is also capable of discriminating between classes of compounds. van Dartel *et al.* (2011c) examined single concentrations of three different phthalates and three different triazoles. They found that analysis of significant effects of single genes was able to discriminate between the two classes of compounds; one of their predefined differentiation tracks (van Dartel *et al.*, 2009a) was also able to discriminate

between the two classes of compounds and to categorize developmental toxicity within the class. The second of their differentiation tracks (van Dartel *et al.*, 2010a) was able to categorize developmental toxicity within the phthalate class but was less useful for the triazole compounds. This may be, in part, because the second differentiation track was developed with exposure to one of the phthalates. The ability of transcriptomics to differentiate within the class of phthalates was further tested (Schulpen *et al.*, 2013). The authors tested various concentrations of four phthalates, three of which are developmental toxicants *in vivo*. The ranking of the compounds based on gene expression data was the same as that obtained when examining NOAELs from *in vivo* studies. The authors concluded that they could correctly rank the potency of these compounds based on the gene expression dose–response data.

3.4 Monitoring Protein Expression

Individual protein expression has also been used as a molecular marker of differentiation. Staining cells with antibodies against the intracellular cardiac markers, α -actinin or myosin heavy chain, followed by fluorescence-activated cell sorting demonstrated that the assay could be shortened from 10 to 7 days, made more quantitative and more objective than the validated assay (Seiler *et al.*, 2004). The modified assay performed as well as the validated assay using 10 chemicals from the list used for the validation study; this modified assay was called the *FACS-EST assay* (Buesen *et al.*, 2009). What has been termed the *adherent cell differentiation and cytotoxicity* (ACDC) assay has been developed by scientists at the EPA (Environmental Protection Agency) to examine a large number of chemicals from their ToxCast™ library. Several modifications to the validated test were made including the use of a different mESC line, plating mESCs without the EB step, and an in-cell western analysis of expression of α - and β -myosin heavy chain along with a DNA stain to determine cell number and cytotoxicity. They first confirmed that their modified assay could predict developmental toxicants by examining three compounds with known developmental toxicant activity (Barrier *et al.*, 2011). The assay was then applied to 309 chemicals from the ToxCast library followed by computational analysis to characterize metabolic and regulatory pathways that may be adversely

affected by environmental chemicals (Chandler *et al.*, 2011). The authors observed significant effects on differentiation or cytotoxicity with a subset of 18% of the chemicals tested. When these data were combined with gene expression data from EPA, the authors noted that multiple genes in reactive oxygen species signaling pathways were associated with decreased differentiation of the mESCs; the *ABCG2* transporter gene was a very strong predictor of decreased differentiation potential.

Proteomic analysis has also been applied to the mEST. Osman *et al.* (2010) used sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis followed by tryptic digestion of proteins and MS/MS analysis. The authors compared proteins from undifferentiated cells as well as from fully differentiated cardiomyocytes. They also used the developmental toxicant, monobutyl phthalate, which inhibits cardiomyocyte differentiation. The authors were able to identify 33 proteins whose expression was altered by monobutyl phthalate. The authors concluded that this proof of principle project could form the basis for further studies to develop a set of proteins that could be used to predict embryotoxicity. This was the goal of Groebe *et al.* (2010), who used dual radioisotope labeling, 2D gel electrophoresis, and MALDI-TOF (matrix-assisted laser desorption/ionization–time of flight) MS for analysis of proteins in cell lysates from cells differentiated under a modified mEST protocol and treated with one of seven either teratogenic or nonteratogenic agents. The common theme of the teratogenic agents was disruption of the proteins in the Ras pathway; the weak or nonteratogenic agents used had no common mechanism, but they did not adversely affect the Ras pathway. The authors planned to verify their findings with additional methods of protein identification and to expand the model by testing with additional compounds. While total proteome analysis holds promise for the identification of altered pathways, it is very labor-intensive and not very amenable to high-throughput analyses. However, identification of a small set of proteins that could be probed by immunological methods may be possible and could be more amenable to high-throughput analysis.

3.5 Monitoring Metabolite Expression

Metabolomic analysis has also been paired with stem cells. West *et al.* (2010) developed a model

using hESCs that were treated with a single concentration of each of 26 chemicals of known teratogenic potential; LC-MS (liquid chromatography–mass spectrometry) analysis was used to determine changes in the abundance of intracellular small molecules following this exposure. A prediction model was established using a subset of 12 of the 26 chemicals tested; the model correctly predicted the teratogenicity status of the additional compounds with 88% accuracy. Although metabolomic analysis is able to monitor multiple metabolic pathways simultaneously, not all of the metabolites can be unambiguously identified. Additionally, the cells in this assay were not differentiated, so it is not clear whether similar results would have been obtained with other human lines. This assay was used to test three concentrations of 11 chemicals from EPA's ToxCast library. The accuracy of the assay was 73%; when the model was retrained including the middle of the three concentrations in the training set, the overall accuracy was increased to 83% (Kleinstreuer *et al.*, 2011).

3.6 Differentiation into Additional Endpoints

The addition of various endpoints to detect effects on other developing organ systems has also been suggested to improve the applicability domain of the test. zur Nieden *et al.* (2004) altered culture conditions to permit differentiation into osteogenic, chondrogenic, and neural cells. They included PCR evaluation of marker genes for each cell type and examined six of the compounds used in the ECVAM validation study. They observed cell-specific effects of several of the compounds that correlated with their *in vivo* teratogenicity. For example, they found that valproic acid, which primarily produces neural tube defects specifically, inhibited expression of neuronal cell genes, while thalidomide specifically inhibited expression of skeletal genes. The nonteratogen, penicillin G, did not alter the expression of any of the genes, while the strong teratogen, 5-fluorouracil, was not specific in its inhibition of gene expression. The authors concluded that the addition of other endpoints could enhance the predictivity of the assay. Festag *et al.* (2007) employed gene expression to evaluate the ability of compounds to interfere with differentiation of mESCs into epithelial cells. When using six of the compounds from all three classes and the prediction

model from the ECVAM validation study, they observed that all six of the compounds were correctly classified and suggested that this modification could be added to the validated mEST to possibly enhance the predictivity of the test.

Differentiation into osteoblasts has been developed by several groups. zur Nieden *et al.* (2010) used a 30-day culture system to differentiate mESCs into osteoblasts. The endpoints tested included alkaline phosphatase activity, calcium content, microscopy with image analysis software to make the assay more quantitative, osteocalcin expression, and cytotoxicity. Cells were treated with penicillin G as a nonteratogen, 5-fluorouracil as a prototypical teratogen, and all *trans* retinoic acid to represent a bone teratogen. Image analysis and calcium content were reproducible endpoints that correctly classified the three chemicals being tested and compared favorably with expression of osteocalcin. Alkaline phosphatase activity correctly classified the three compounds but was more variable. The authors then used this assay to test the embryotoxic potential of four chloride compounds with increasing valence (zur Nieden and Baumgartner, 2010). They applied several different statistical methods to categorize the compounds with slightly different results depending on the method used. For example, when applying the ECVAM prediction model, aluminum chloride was classified as weakly embryotoxic with the other three compounds (sodium chloride, lithium chloride, and magnesium chloride) being classified as nontoxic. Lithium chloride was classified as weakly embryotoxic in the validated mEST (Genschow *et al.*, 2002) and was used as a positive control in the *ReProGlo* assay because of its effects on the canonical Wnt pathway (Uibel *et al.*, 2010). A linear correlation method classified lithium chloride and aluminum chloride as embryotoxic, and a logarithmic categorization classified all of the compounds as nonembryotoxic. The authors concluded that lithium chloride, sodium chloride, and aluminum chloride might have adverse effects on osteoblast differentiation. Dienelt and zur Nieden (2011) also examined the effect of high levels of glucose on differentiation into osteoblasts. They found that high glucose concentrations decreased osteoblast formation as detected by morphology, specific calcium staining, gene expression, and decreased alkaline phosphatase activity. In addition, the authors observed fewer osteoclasts in the high

glucose cultures. The differentiation protocol produces not only osteoblasts but the stem cells also differentiate into osteoclasts.

de Jong *et al.* (2012) developed a protocol to differentiate mESCs into osteoblasts over a 21-day culture period and used this protocol to test the embryotoxicity of six different chemicals. Endpoints evaluated included mineralization (as determined by calcium content) and expression of three different osteogenic genes. The authors observed increases in calcium content and decreases in gene expression for most of the compounds tested (five of the six compounds were embryotoxic). They compared these results with the validated mEST and concluded that the effective concentrations of the six compounds were very similar in the two assays and that little was to be gained by adding differentiation into osteoblasts to the mEST.

Another popular differentiation endpoint is neuronal cells. Baek *et al.* (2011b) developed a culture system that differentiated mESCs into neuronal cells as measured by morphology, gene expression, and flow cytometry. They tested this system with two neurotoxicants, lead(II) acetate and Aroclor 1254; they compared the results of the validated mEST to their modified culture method. The validated mEST predicted that lead(II) acetate would be nonembryotoxic and Aroclor 1254 was weakly embryotoxic. Using either flow cytometry or real-time PCR analysis of *Map2*, a neuron-specific marker, as the endpoint of neuronal differentiation, the authors found that both compounds were now predicted to be weakly embryotoxic using the ECVAM prediction model. They later refined the culture system and examined flow cytometry of Tuj1, an early neuronal marker, and real-time PCR analysis of *Map2* as the differentiation endpoints (Baek *et al.*, 2012). Lead(II) acetate and Aroclor 1254 were again tested as neurotoxicants and penicillin G was tested as a nonembryotoxicant in three independent laboratories. In the mEST, Aroclor 1254 was predicted to be weakly embryotoxic, and lead(II) acetate and penicillin G were nonembryotoxic. However, using the neuronal assay, both lead(II) acetate and Aroclor 1254 were predicted to be weakly embryotoxic, while penicillin G was predicted to be nonembryotoxic. The authors further tested this refined culture system with additional neurotoxicants, some of which had been misclassified as nonembryotoxic by the validated mEST (Baek *et al.*, 2011a). The authors tested the neurotoxicants methyl mercury,

valproic acid, sodium arsenate, and sodium arsenite as well as the nonembryotoxicants saccharin, isoniazid, and ascorbic acid. Using either flow cytometry of Tuj1 or PCR to monitor *Map2* in the ECVAM prediction model resulted in the classification of methyl mercury and sodium arsenite as strong embryotoxicants with sodium arsenate and valproic acid classified as weakly embryotoxic; the three nonteratogenic compounds were all correctly classified. The authors concluded that this assay could identify developmental neurotoxicants including some compounds that would be misclassified in the validated mEST.

Theunissen *et al.* (2010) developed a 13-day culture system that resulted in the differentiation of neuronal cells; cell types of both the endoderm and mesoderm lineages were present as well as ectodermal cells. Both GABA (γ -aminobutyric acid) ergic and glutamatergic neurons were present in the culture system, as well as glial cells; however, no oligodendrocytes were observed with immunostaining for two different markers. When methyl mercury was tested in this system, it was found that the number of undifferentiated and neural precursor cells increased with increasing concentrations of methyl mercury. Methyl mercury was misclassified in the validated mEST (Genschow *et al.*, 2004) but was correctly classified in an early neuronal version of the mEST (Stummann, Hareng, and Bremer, 2007). In this newer culture system (termed *ESTn*), methyl mercury decreased neurite outgrowth morphologically, but this was not reflected in the flow cytometric analysis of β III-tubulin, a neuron-specific marker. They found altered gene expression when analyzing the transcriptomic changes induced by methyl mercury (Theunissen *et al.*, 2011). When expanded to a diverse group of developmental toxicants, these compounds increased cytotoxicity and decreased neural differentiation (Theunissen *et al.*, 2012b). In spite of similar morphological outcomes, these toxicants altered different sets of genes; this may be explained, in part, by the differences in proposed mechanisms of action of the compounds. They also found concentration-dependent effects on gene expression with a different set of compounds (Theunissen *et al.*, 2012a). With all of these compounds, transcriptomic analysis could provide information on the mechanism of toxicity of the compound. Combining all of their data on transcriptomic changes in response to various chemicals resulted in a gene set (*ESTn_enriched* only) of 100 genes

with 74% prediction accuracy and a restricted set (*ESTn_restricted*) of 29 genes with 84% accuracy (Pennings, Theunissen, and Piersma, 2012).

3.7 Use of hESCs

One of the goals of the NRC's "Toxicity Testing in the 21st Century: A Vision and a Strategy" (National Research Council (NRC), 2007) is the use of human cell lines to eliminate cross-species extrapolation. The use of human cells in the mEST has proven to be quite challenging due, in part, to complex culture conditions (Seiler *et al.*, 2011). As mentioned earlier, there are still ethical concerns regarding the use of hESCs, and additional quality control information on the reprogramming process will be needed if iPSCs are used (Vojnits and Bremer, 2010). Problems with reprogramming include low reprogramming efficiency, partial reprogramming, genetic instability, and the "epigenetic memory" of the reprogrammed cells as well as differences dependent on the type of cell used and the method of reprogramming (reviewed by Wobus and Löser, 2011). There has been one attempt to use hESCs in a human version of the EST. Adler *et al.* (2008) used the hESC line H1 and MRC-5, a human embryonic lung fibroblast line as the differentiated line. They tested various culture conditions, and although beating cardiomyocytes were visualized, there was a great deal of variability in this endpoint. The authors then chose to monitor gene expression and observed decreased expression of pluripotency markers and increased expression of cardiac markers. Although the assay showed some promise, much more work will be needed to develop a robust human assay. West *et al.* (2010) also used hESCs in their system, which examined metabolomic responses of the cells to various developmental toxicants. However, their assay did not assess the effects of the compounds on the ability of the cells to differentiate. More work will be needed before human cells can be used in this assay system (see Chapter 16).

4 NEUROTOXICOLOGY AND DEVELOPMENTAL NEUROTOXICOLOGY

Advances in our understanding of stem cell biology and neuroscience have opened up new avenues of research for detecting early-life stress-induced

neurotoxicity and developing potential protection/prevention strategies against toxicant-induced neuronal injuries (Kiss *et al.*, 1994; Wang, Rougon, and Kiss, 1994; Wang *et al.*, 1996; Brokhman *et al.*, 2008; Trujillo *et al.*, 2009). Stem cell biology, when exploited along with genomics, proteomics, or metabolomics, can be utilized to enhance the understanding of complex biological processes such as apoptosis and can provide fundamental concepts necessary for constructing models of the building blocks of biological systems during development. As these models evolve and become linked together as integrative modules, they provide the components necessary for use in a developmental biology/toxicology approach.

The developing nervous system can vary dramatically in susceptibility to neurotoxic insults depending on stage of development. Because of the complexity and temporal features of the manifestations of developmental neurotoxicity, this area of study may benefit greatly from stem cell models. A major goal of applying a stem cell biology approach to developmental neurotoxicology is to predict the functional outcomes of component-to-component relationships using integral *in vitro* models that allow for the directional and quantitative description of responses at the cellular level that occur as a result of perturbations of its component systems. These perturbations may come in the form of environmental alterations or exposures to drugs, other chemicals, or infectious agents. Thus, the application of stem cell biology/models toward understanding issues relevant to developmental neurotoxicology has the potential to advance our understanding of brain-related biological processes, including neuronal plasticity and toxicity (Keirstead *et al.*, 2005; Li *et al.*, 2005; Lamba *et al.*, 2006; Lee *et al.*, 2006; Kang *et al.*, 2007).

Neural stem cells and neural progenitor cells are present throughout development and persist in the adult nervous system. They were first isolated from the embryonic and adult mouse striatum in the early 1990s and have since been identified in nearly all regions of the embryonic mouse, rat, and human central nervous systems (CNSs) (Keirstead *et al.*, 2005; Molero *et al.*, 2009) as well as the subgranular layer of the dentate gyrus in the mature CNS. Recently, malignant multipotent neural stem-like cells, or brain tumor stem cells, have also been isolated from brain cancers.

Neurogenesis is an important process for nervous system development and maintenance. During neurogenesis, neural stem cells generate neural progenitors that mature into functional neurons. Pharmacological enhancement of neurogenesis represents a potential therapeutic approach for the treatment of neuronal loss in neurodegenerative diseases, such as stroke, brain damage, Parkinson's disease, and Alzheimer's disease. Accordingly, there is great interest in using neural stem cells during early drug development as a screening tool for neurogenic compounds.

It is not possible to thoroughly explore the effects of an early-life stress, such as the effect of prolonged exposure to anesthetic agents on neurons *in vivo* in human infants or children. The availability of stem-cell-derived models, especially human embryonic neural stem cells, along with their capacity for unlimited proliferation and potential to differentiate, has provided a potentially invaluable tool for examining the developmental effects of anesthetic agents *in vitro*. Preclinical research models such as neural stem cells can inform clinical interventions and vice versa. Thus, a thorough characterization of neural stem cells and a better understanding of neural patterning and the generation of the three major cell types that constitute the CNS (neurons, astrocytes, and oligodendrocytes), as well as the microenvironments that can support them, is crucial for increasing the likelihood of clinical success of these therapeutic agents. Representative general anesthetic drugs (e.g., ketamine, propofol, and related compounds) are examples to delineate how stem cells can be employed to solve important problems in developmental neurotoxicology. Several advantages of using stem cells as models for addressing critical issues related to the toxicity of pediatric anesthetics are discussed. These include the relationships between drug-induced neurotoxicity and how stem cells can be used as tools for dissecting out mechanisms underlying pharmacological and toxicological phenomena during development. The discussion focuses on representative general anesthetics as an example of how stem cells can be valuable for identifying the doses and time course over which individual drugs cause damage and/or protect neural stem cells and cells derived from them, change their proliferation rate, and alter their fate (differentiation into neurons, oligodendrocytes, and astrocytes) *in vitro*. Taken together, stem-cell-derived models can be one of the best systems in evaluating adverse

health impacts of toxicants during development because (i) specific cell types, such as neurons, astrocytes, or oligodendrocytes can be tested for adverse effects *in vitro*; (ii) neural stem cells taken from animals require only a minimal number of animals; and (iii) the ability to assess the brain's ability to regenerate following exposure to a toxic compound during development can be examined. Therefore, by examining the potential toxic effects of some general anesthetic agents as an example, a strategy can be defined to use embryonic neural stem cell cultures for monitoring neural stem cell expansion, proliferation, and differentiation.

As an example of the utility of stem cells for evaluating neurotoxicity, the anesthetic agent propofol was used in a series of experiments involving rat embryonic neural stem cells harvested from the cortical tissue of embryonic day 14 Sprague-Dawley rats. Propofol is a GABA receptor agonist used as a pediatric anesthetic agent that has been shown to cause neuronal death in brains of experimental animals (Yu *et al.*, 2013). Preliminary work from one of our laboratories demonstrated that a 24-h exposure to propofol induced a dose-related increase in toxicity in rat neural stem cells in culture. This toxicity appeared to be due to an increase in apoptotic cell death. Elevated levels of 8-oxo-2-deoxyguanosine suggested that the mechanism might be related to increased generation of reactive oxygen species, and the administration of acetyl-L-carnitine effectively blocked some of the toxicity of the anesthetic (C. Wang, unpublished observations).

While neurogenesis has been shown to be involved in learning and memory processes, the incorporation of embryonic neural stem cell models, especially human neural stem cells, into the process of identifying toxicities and underlying mechanisms is of considerable interest for determining (i) how neural stem cells respond to stress (e.g., prolonged anesthetic exposure); (ii) how their fate (e.g., differentiation from progenitor cells into neurons or glia) may be affected by anesthetic exposure; (iii) how anesthetic exposure directs or signals these cells to undergo apoptosis or necrosis; (iv) how anesthetic exposure triggers mitochondrial dysfunction; (v) how antioxidant agents and knockdowns of specific transcription modulators or receptors [e.g., NMDA (*N*-methyl-D-aspartate) receptors] may affect anesthetic-induced neurotoxicity; and (vi) whether single or combined anesthetics enhance toxicity or protect neuronal cells from toxicity.

Human neural stem cells could be used as a test system to determine whether toxicant-induced nerve cell damage observed in some preclinical (rodents or nonhuman primate) studies is relevant to human fetuses. The ability of neural stem progenitor/precursor cells to self-renew and differentiate into tissue-appropriate functional cell types has generated intense scientific interest and the hope of new therapies for neurological diseases (Temple, 2001; Arvidsson *et al.*, 2002; Nakatomi *et al.*, 2002). Therefore, the use of neural stem cell models, especially those of human origin, holds promise for helping elucidate relevant mechanisms underlying the etiology of neurotoxicity associated with developmental exposures to toxicants and may also help identify avenues of protection or prevention. Thus, the study of neural stem cells may provide the opportunity for assessing the brain's own regenerative capacity after experiencing events related to accidental exposures to harmful substances, overdoses, or prolonged exposures to drugs including some general pediatric anesthetics or environmental chemicals, deliberate poisonings, and the unintended adverse consequences associated with chemical or drug mixtures and, presumably, other compounds. In addition, genetic differences, sex, developmental stage, and species differences in response to toxicants have provided a relatively solid foundation of knowledge for the science of toxicology (Kang and Trosko, 2011).

5 SUMMARY

It is clear that stem cells have a role to play in developmental toxicology and neurotoxicology. Stem cells can provide evidence on mechanisms of action of compounds, and this information can be very important on a compound-specific basis. However, such mechanistic information may not be useful in a high-throughput assay. The mEST has been proposed as an *in vitro* test that can be useful in predicting the embryotoxicity of chemicals and in prioritizing chemicals for further whole animal testing (see Chapter 16). Modifications have been proposed to the validated version of the test to increase its applicability domain and to enhance its predictivity. The replacement of microscopic evaluation of beating cardiomyocytes by quantifiable and objective molecular endpoints would enhance the test. Additionally, the test covers only a small period of

embryonic development; the addition of endpoints of later embryonic development, such as osteoblasts and neuronal cells could increase predictivity of the test (Riebeling *et al.*, 2012) and move it into the arena of developmental neurotoxicity. *In vitro* toxicity tests using stem cells are currently evolving, as are the future regulatory roles of such tests.

DISCLAIMER

This chapter reflects the views of the authors and should not be construed to represent FDA's (Food and Drug Administration) views or policies.

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Human Stem-Cell-Derived Cardiomyocytes in Drug Discovery and Toxicity Testing

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1 INTRODUCTION—NEED FOR HUMAN CARDIOMYOCYTES IN DRUG SCREENING

Cardiac side effects of various drugs have been the primary causes of drug withdrawals over the past 10 years (Valentin and Hammond, 2008). This fact indicates that the systems applied by the drug developers for cardiotoxicity testing have been in several cases inadequate, although identification of potential cardiotoxicity of candidate drugs at an early phase in the drug discovery process is a key objective for the pharmaceutical industry.

Preclinical toxicity studies often use *in vitro* and *in vivo* animal models such as mouse, rabbit, or dog. Owing to the existing species differences [e.g., in ion channel drug sensitivities (Jost *et al.*, 2005; O'Hara and Rudy, 2012; Redfern *et al.*, 2003; Rudy *et al.*, 2008) and cAMP (cyclic adenosine monophosphate) and cGMP (cyclic guanosine monophosphate) phosphodiesterases (Johnson *et al.*, 2012)], possible cardiac side effects that may occur in the human patients could not be excluded in many cases. Test systems, such as tumor-derived or immortalized mammalian cell lines, ectopically

expressing ion channels or receptors characteristic for the human heart, are often employed. However, similarly to isolated tissues (e.g., perfused animal hearts or primary cell cultures), these are often unreliable in mimicking a functional human system. Thus, these models provide only limited information about possible effects of a particular compound on the normal human heart. Reliable cardiac pharmacology and toxicity tests clearly require the application of human cells, and as far as possible, human cardiomyocytes (CMs).

The application of human cardiomyocyte-based test systems could facilitate not only the examination of severe cardiotoxic side effects of drugs but also the discovery of potential medications for heart-specific diseases. Clearly, many new chemical entities have failed in early preclinical studies, because drug targets identified and validated by currently available test systems proved to be non-predictive when translated into humans (Ferri *et al.*, 2013).

The scarce availability of human cardiac tissues hinders the use of primary cardiomyocyte cultures in *in vitro* drug assays. Human embryonic stem

cell (hESC) lines offer an advantage over these limited resources, as hESCs possess the properties of self-renewal and directed tissue differentiation. Accordingly, hESCs are able to go through numerous cycles of cell division while maintaining an undifferentiated state, whereas based on their pluripotency, they are able to differentiate into any specialized cell types of the human body. Because of the abilities of unlimited expansion and differentiation potential, hESCs are potentially the most appropriate sources for generation of human CMs. However, ethical considerations may restrict their use, as hESC lines are derived from the inner cell mass of human blastocyst-stage embryos.

An alternative source of human CMs has been provided by the generation of induced pluripotent stem cells (iPSCs), which can be obtained by reprogramming of human somatic cells with transcription factors into an embryonic stem cell (ESC)-like undifferentiated state. This method was first described by Takahashi and Yamanaka (2006) and the importance of their work was recognized by the Nobel Prize in 2012. An inherent problem of using either hESCs or iPSCs for the generation of CMs may be that the adult-like forms of CMs are not reached during differentiation, and only the fetal type of cardiac cells can be obtained. This possibility requires an appropriate examination of the molecular components of the pluripotent stem-cell-derived tissues and development of differentiation methods toward adult phenotype.

Patient-specific genetic polymorphisms may result in variable responses to pharmacological agents, which cannot be considered by using the currently available systems. One of the most fascinating advantages of the iPSC technology is that it allows the generation of patient-specific CM, hereby advancing the concept of personalized medicine. Moreover, not only patient-specific but also disease-specific cellular assays can be provided for drug development and safety assessments based on patient-specific iPSC-derived CMs.

In this chapter, the current use of human stem-cell-derived and reprogrammed CMs for drug discovery and cardiotoxicity testing is discussed. We also review the validity of using human CMs in drug screening and highlight the potential shortcomings that have to be overcome for their wider application in pharmacology.

2 GENE DELIVERY AND STABLE GENETIC MODIFICATIONS OF STEM CELLS

Stem cells in general are excellent model systems for investigating tissue differentiation processes, and in the meantime, they also provide invaluable platforms for *in vitro* drug screening methodologies. For such purposes, it is often required to establish stem cell progenitors with stable genetic modifications, without disturbing their pluripotent or multipotent differentiation potential. Currently available gene delivery techniques, however, may not be suitable for all types of stem cells, and their efficiency values versus their invasive characteristics should always be carefully considered *a priori* their applications. In this section, we overview the most widely used gene delivery methods, discussing their applicability, especially aiming at establishing *in vitro* differentiation toolkits.

Considering gene delivery efficiency, viral-based methods are undoubtedly the methods of choice when stem cells that express particular transgenes are required. Retroviral systems, especially the group of lentiviral-based gene delivery vehicles, are extremely efficient and robust methods and therefore are widely used for such purposes. However, their unfavorable genomic integration profiles could be a strong argument against their usage; the delivered transgene is often inserted into genomic loci of actively transcribed genes, increasing the risk of insertional mutagenesis (Schroder *et al.*, 2002; VandenDriessche, Collen, and Chuah, 2003). Unfortunately, this nonrandom integration profile is usually the most distorted toward active genomic regions in the case of the most efficient viral systems [such as the human immunodeficiency virus (HIV)-like gene delivery vehicles], which otherwise offer valuable characteristics including the ability to transduce even nondividing cell types (such as some dormant stem cell types). The concern of nonrandom integration is most expressed in case of gene therapy methodologies; however, insertional mutagenesis potentially change gene expression profiles and therefore the overall cellular phenotypes can also be unfavorable when establishing *in vitro* cellular screening systems. Such issues prompted research scientist to apply nonintegrating viral vector types, such as adenoviruses and their related counterparts (Kron and Kreppel, 2012). These viruses are certainly useful when a transient expression of the transgene is sufficient; however, stem cells to

be used as drug screening tools definitely require long-term stable expression profiles.

Another problem with using viral-based methodology is that stem cells possess strong defense mechanisms against such invasive genetic elements. These include the RNA interference phenomenon, more specifically the endogenous siRNA (small interfering ribonucleic acid) or the piRNA (piwi-interacting ribonucleic acid) pathways, which can be activated regardless of the virus types (Golden, Gerbasi, and Sontheimer, 2008; Ishizu, Siomi, and Siomi, 2012). However, additional defense mechanisms can specifically target viral-like promoters such as the widely used cytomegalovirus (CMV) promoter types. Such promoter silencing can also occur when using endogenous housekeeping promoters [e.g., phosphoglycerate-kinase (PGK) or elongation factor-1 alpha (EF1 α) promoters], and the unpredictable and cell-type-specific magnitude of this phenomenon impose problems on the efficiency of transgene expression (Chan *et al.*, 2008; Chung *et al.*, 2002; Liew *et al.*, 2007; Xia *et al.*, 2007). Some related promoter issues concerning reporter systems used in CMs are also discussed in Section 6.

To overcome the undesired effects related to the viral-based methods, other “traditional” gene delivery techniques have been applied with variable success rates. This usually involves expressing the given transgenes from episomes, such as plasmid expression vectors. Apart from providing only transient expressions (random integration might occur with low efficacy and with questionable stability), the serious drawback of such an approach is the low efficiency of transfection by which these expression cassettes are delivered into the target cells. Lipid-based transfection technology could provide reasonable delivery efficiency with low side effects such as cell mortality. However, efficiency may decrease if cells are present in aggregates, which is often the case using ESCs that usually grow in clumps; therefore, some protocols might require separating the aggregates into single cells. As opposed to murine ESCs, however, human counterparts do not tolerate separation so well, which further decreases survival rates, thereby also lowering transfection efficiency. Other methods such as electroporation, including a new variant called nucleofection, are promising techniques to increase transfection efficiency tremendously, although cell mortality may also increase

in case of certain cell types (Mellott, Forrest, and Detamore, 2013). Nevertheless, the transient expression profile still remains a serious drawback; therefore, other approaches with stable genomic integration are required to develop genetically modified stem cells with robust *in vitro* screening potential.

DNA transposon-based gene delivery technology for mammalian cells has been recently developed and successfully applied in various model systems, and it is now considered to represent a comparably efficient alternative to viral-based approaches (Ivics and Izsvak, 2006). The first such developed platform was the *Sleeping Beauty* (SB) system, a “resurrected” artificial *Tc-1/Mariner* type transposon, which truly revolutionized the field of gene delivery and also gene therapy (Ivics *et al.*, 1997; Izsvak and Ivics, 2004). Since its original development, several lines of progress have further refined this technology, including the use of other artificial transposons [e.g., *Frog Prince* (Miskey *et al.*, 2003)], exogenous transposons from other species [*piggyBac* (PB) (Ding *et al.*, 2005) and *Tol2* (Balciunas *et al.*, 2006)], and the improvement of the existing systems by developing hyperactive versions of the transposase proteins (Zayed *et al.*, 2004). The development of the SB100x hyperactive version (Mates *et al.*, 2009) was considered to be a new milestone in gene delivery and therapy protocols, as exemplified by its selection as the molecule of the year by the prestigious Science magazine (<http://www.biotechniques.com/news/Sleeping-Beauty-named-Molecule-of-the-Year/biotechniques-187068.html?autnID%BC191663>). Nowadays, the SB100x and the PB transposon platforms are the most efficient gene delivery techniques of this kind and are widely used in various applications, from genetic screens to potential gene therapy applications. PB is sometimes favored because of its ability to tracelessly remove its delivered cargo, which may have special importance in the generation of pluripotent stem cells by reprogramming factors (Woltjen *et al.*, 2009). However, just like many other transposon systems, PB has an unfavorable integration profile with the preference of actively transcribed genes, and similarly to viral-based applications, this propensity may increase the risk of insertional mutagenesis (Grabundzija *et al.*, 2010). The SB system, on the other hand, is the only known gene delivery system so far with the most random integration profile, and because of the total absence of similar elements in the human

genome, this technology is now considered to be the safest available application for gene delivery. These characteristics certainly played a significant role in choosing SB for gene therapy applications and formed the basis to initiate a clinical trial using this methodology (Williams, 2008).

The transposon platforms are able to overcome some difficulties presented by the viral-based delivery systems, including the need for special, dedicated laboratories for the use of potentially hazardous viruses. Nevertheless, the question of transgene integration profile still remains a crucial issue. Even if the risk of mutagenesis is lowered, the neighboring genomic loci could strongly influence the expression of the integrated transgene (position effect variation). This already raised the need for targeting the transgenes into genetically isolated and “harmless” loci, not to mention the desired exchange of endogenous gene alleles for other variants in order, for instance, to establish cell lines representing given disease models. In the mouse genome, the so-called Rosa26 region is a favored locus for targeting a specific transgene, as it does not seem to influence the expression profile of other endogenous loci and *vice versa*; the expression is isolated from the neighboring chromosomal regions, providing the desired and reliable transgene expression level. There are similar regions also in the human genome but they are less characterized yet. Besides, for long time, the rare event of homologous recombination remained the only possibility to specifically insert the gene of interest into a given genomic locus. Recently, however, emerging new genome editing technologies made targeted transgene integration widely available in various genomes, namely by the use of new modular nuclease enzymes.

The first example of such molecular tools was the zinc finger nucleases (ZFNs) that were demonstrated to target specific genomic loci first in *Drosophila* (Bibikova *et al.*, 2002) and later in the human genome (Porteus and Baltimore, 2003). The zinc finger motif is a very common module of transcription factors, providing the specificity of DNA binding of these regulators. Fusing such a motif with the nuclease domain of the Fok I restriction endonuclease enables the user to create DNA cleavage sites in specific regions, thereby creating targeted mutations in desired loci. The so-formed double-stranded breaks (DSBs) activate the inner DNA repair mechanisms of the cell, which can be utilized to insert given sequences into the

targeted region. If we simultaneously provide a double-stranded DNA with flanking sequences of the DSB, it will be used as a template to repair the damage, creating the desired insertion. Differently engineered zinc finger motifs can be used to change the specificity of ZFNs, which makes this system a really versatile platform for genome editing of any cell types, including stem cells (Maeder *et al.*, 2008). Another group of enzymes called *meganucleases* may also be used for such purposes (Cabaniols and Paques, 2008). These represent “homing endonucleases” derived from mobile genetic elements, with strong specificity for given DNA sequences. Because of their evolutionary origin within the cells, their advantage is that they seem to be less toxic than ZFNs. However, unlike ZFNs, their structure is not modular; so, it is more difficult to “reengineer” them in order to change their target specificity. Recently, another group of useful targeting tools were identified from an unexpected source, namely from a bacterial plant pathogen group called *Xanthomonas*. These so-called transcription activator-like effector nucleases (TALENs) have a unique structure; they are built up of 34 amino acid repeats in a modular manner, and these small structural motifs seem to have specific binding capacity to single nucleotides of DNA. This “binding code” looks even more flexible to engineer than that of ZFNs, and fusing these again with the nuclease domain of Fok I restriction endonuclease represents a very useful molecular alternative for ZFNs (Bogdanove and Voytas, 2011). Further investigations are still required to test the safety and applicability of any of these platforms, especially considering the potential applications in various types of stem cells.

With the unprecedented speed of developing new molecular tools, there are already various techniques available both for gene delivery and for targeted genome editing of eukaryotic cells (Table 1). Careful combination and optimization of these methodologies will truly provide stem-cell-specific toolkits to generate useful platforms for research purposes in the future. In the next sections, we examine how these approaches have already converged to investigate a specific differentiation pathway, the cardiac differentiation pathway from stem cells. Apart from bearing scientific significance, these studies undoubtedly also have strong influence in the field of regenerative medicine.

Table 1. Some important aspects of the discussed gene delivery systems.

Gene delivery methods	Special requirement for delivery	Transgene integration profile	Overall efficiency of transgenes	Special laboratory requirements	Genomic defense
Episomes (plasmids)	Yes, transfection	Low frequency of integration, often not stable	Low	No	Usually not present
<i>Viral-based systems</i>					
Adenoviral type	No	No integration	Only transient	Yes	Yes (promoter inactivation, RNAi)
Retroviral type	No	Preference for active gene loci	Very high	Yes	Yes (promoter inactivation, RNAi)
<i>Transposon-based systems</i>					
Sleeping beauty	Yes, transfection	Random	High	No	None detected
PiggyBac	Yes, transfection	Preference for 5' gene regions	High	No	None detected
Others (Tol2, etc.)	Yes, transfection	Often preferring active gene loci	Moderate	No	None detected

3 GENERATION OF HUMAN CARDIOMYOCYTES

3.1 Cardiomyocyte Generation from Pluripotent Stem Cells

Human pluripotent stem-cell-derived CMs may be applied to study disease-specific cellular alterations, to screen new drugs, as well as in regenerative medicine and cell therapy. While therapeutic applications may still have to overcome several barriers, one of the most important practical use of human CMs might be in cardiotoxicity applications and cardiac drug discovery (Figure 1).

In contrast to cells in numerous tissues, such as the skin or the liver, the key cells in the heart are practically not dividing or regenerating; therefore, less than half of the CMs are exchanged during a normal lifespan (Bergmann *et al.*, 2009).

Cardiac repair is therefore only possible if the low numbers of endogenous cardiac progenitor cells are stimulated to divide or new CMs are transplanted into the heart.

The two major types of human pluripotent stem cells, the embryonic and the iPSCs, are both able to differentiate into any cell types of the human body, including CMs (Ameen *et al.*, 2008). hESCs have been isolated from the inner cell mass of the blastocyst-stage embryos generated in *in vitro* fertilization processes (Bongso and Tan, 2005; Gepstein, 2002; Thomson *et al.*, 1998). The first hESC lines

were established in 1998 (Thomson *et al.*, 1998). Almost 3 years later, the first report of CMs derived from hESCs was published (Kehat *et al.*, 2001). By now, hundreds of hES cell lines are available for research applications, and numerous methods have been published on their culturing and directed differentiation (Denning *et al.*, 2006; Vidarsson, Hyllner, and Sartipy, 2010). However, the ethical concerns regarding the use and/or destruction of human embryos during hES cell line derivation reduces the general applicability of these cell types. Moreover, in therapeutical applications, additional two major obstacles are associated with the application of hESCs—the risk of immune rejection after transplantation and the potential formation of teratoma from the remaining pluripotent cells.

The human induced pluripotent stem cells (hiPSCs) may provide another major source of *in vitro* differentiated CMs. In 2006, Takahashi and Yamanaka published an article on the generation of iPS by retrovirally introducing four transcription factors (also known as *Yamanaka factors*) into mouse fibroblasts (Takahashi and Yamanaka, 2006). A year later, the generation of human iPS cells was successfully achieved by several groups (Takahashi *et al.*, 2007; Yu *et al.*, 2007). By now, a variety of cell sources, different combinations of transcription factors, and different delivery techniques have been successfully used for generating iPS cells. These two types of pluripotent stem cell lines have numerous similarities in morphology, maintenance,

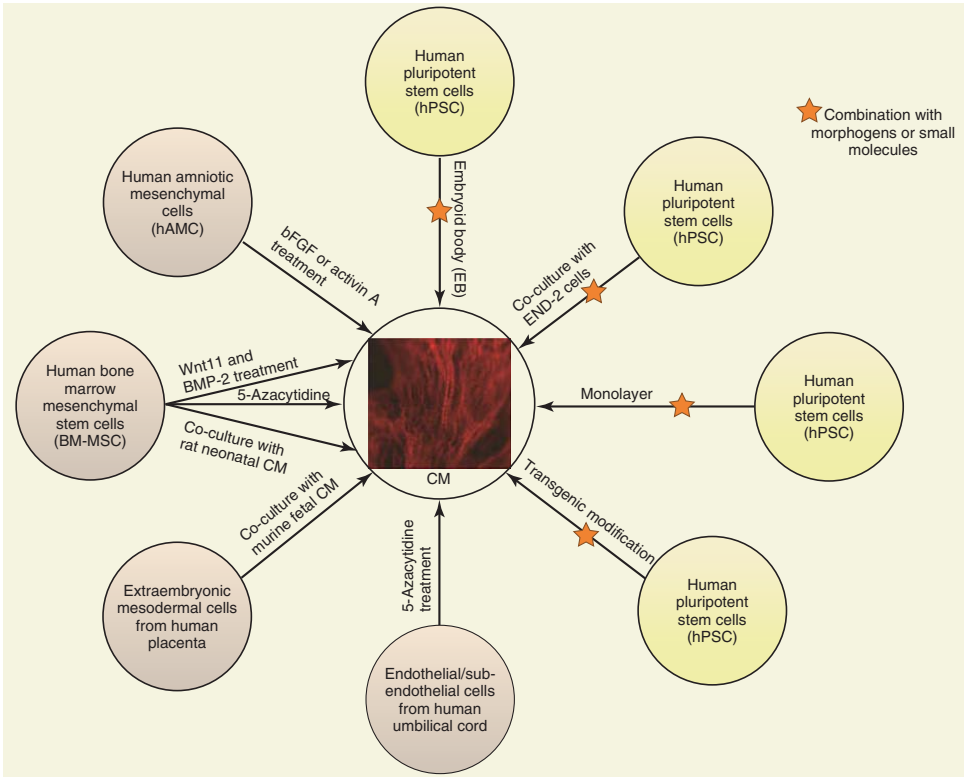


Figure 1. Potential methods to generate human cardiomyocytes.

and differentiation potentials. In general, they can be cultured for theoretically unlimited numbers of cell divisions and yield large number of equally pluripotent cells, with unlimited differentiation potency.

Several protocols have been described for generating CMs from human pluripotent stem cells. The “classical” method to induce cardiomyocyte differentiation is the embryoid body (EB) formation in suspension. These multicellular three-dimensional aggregates are formed when hES cells are cultured without feeder cells in suspension. EBs may mimic the complex cell–cell interactions during early embryonic development. The addition of bovine or human serum to the media for several days induces differentiation into cell types of all three germ layers, including contracting CMs (Kehat *et al.*, 2001; Xu *et al.*, 2002). For cardiomyocyte generation, after few days of growth in suspension, EBs are plated on gelatin-coated dishes. Some of the EB-outgrowths start spontaneously contracting (beating) within a few days post-plating. This autonomous and highly stochastic differentiation method typically results in a small percentage of CMs, among various other differentiated cell types, within the EBs.

Numerous protocols have been developed to increase differentiation efficiency toward the cardiac lineage. On the basis of the cardiac inductive activity of endoderm in early embryonic studies, Mummery *et al.* designed a coculture of hESCs with END2, a mouse visceral endoderm-like cell line (Mummery *et al.*, 2002, 2003; van den Eijnden-van Raaij *et al.*, 1991). END2 cells were shown to support cardiac differentiation through secretion of prostaglandin I₂ (Xu *et al.*, 2008b). A 24-fold increase could be observed in cardiomyocyte yield using hES2–END2 cocultures when serum-free media was used instead of 20% fetal calf serum (FCS)-containing media (Passier *et al.*, 2005), and this differentiation efficacy was further enhanced by ascorbic acid. This method can be applied to several stem cell lines including those that are not able to differentiate through EB formation.

An alternative method for cardiac differentiation is based on a monolayer model, described by Laflamme *et al.* (2007). In this protocol, hESCs are cultured in a feeder-free system and induced to differentiate into CM by a serum-free medium, supplemented with defined growth factors, for example, BMP4 (bone morphogenetic protein) and activin

A. Both factors are members of the TGF β (transforming growth factor-beta) family that is known to be involved in multiple aspects in embryonic development, including the induction of mesoderm and the promotion of cardiogenesis (Paige *et al.*, 2010). These differentiation methods have been suggested to be dramatically more efficient than the classical EB-based methods (Laflamme *et al.*, 2007).

Pluripotent stem cell differentiation is significantly modulated by growth factors or morphogenes, which can be applied to direct the differentiation of hESCs into the cardiac lineage (Xu, 2012). Three major families of growth factors, the members of the transforming growth factor β -family (BMPs and activin), the Wnt/ β -catenin signaling, as well as the fibroblast growth factor (FGF) family, have been discovered to be expressed in the early endoderm and are among the most important regulators of cardiac development (Filipczyk *et al.*, 2007; Kwon *et al.*, 2009).

Numerous small molecules that target the cardiogenesis pathway have been tested *in vitro* and found to enhance cardiomyocyte differentiation from hPSC. 5-Azacytidine (a demethylating agent) (Gai *et al.*, 2009; Wang *et al.*, 2010; Xu *et al.*, 2002; Yoon *et al.*, 2006), cyclosporin-A (Fujiwara *et al.*, 2011), or ascorbic acid (Wang *et al.*, 2010) have been also reported to possess pro-cardiogenic effect. For example, treatment of hESC with 5'-deoxyazacytidine resulted in an increase in cardiac differentiation and upregulated the expression of cardiac-myosin heavy chain. Another possibility to guide the differentiation of the hESCs toward cardiac lineage is transgenic modification. Transduction of four transcription factors, *GATA4*, *TBX5*, *NKX2-5*, and *BAF60c* was shown to be essential to generate cardiac progenitors and beating CMs from the HUES 7 hESC line (Dixon *et al.*, 2011). Single factors alone were not capable to induce cardiomyocyte differentiation of hESCs in contrast to murine stem cells (David *et al.*, 2009; Grepin, Nemer, and Nemer, 1997; Hu *et al.*, 2010). To detect formation of CMs, the HUES 7 hESC line was genetically modified to express the monomeric red fluorescent protein (mRFP) under the control of the cardiac-specific MYH6 promoter. Electrophysiological analysis of differentiated CMs by patch clamp showed action potentials (AP) resembling atrial and ventricular cardiomyocyte subtypes. Fonoudi *et al.* (2013) demonstrated that the direct delivery

of recombinant Islet1 (ISL1) protein enhanced the overexpression of its target genes, induced hESCs differentiation into cardiomyocyte phenotype, and resulted in up to a threefold increase in the number of beating areas. The number of cells that expressed cardiac-specific markers (*CTNT*, *CONNEXIN 43*, *ACTININ*, and *GATA4*) doubled and the expression level of *NKX2-5* increased after overexpression of Is1 in hESCs. This protocol was also reproducible for another hESC lines. Despite increasing effectiveness of differentiation protocols, some technical challenges should be solved and standardized protocols are needed for establishment of reliable drug screening platforms.

3.2 Cardiomyocyte Generation from Somatic Stem Cells

As discussed earlier, generation of human cardiac cells from pluripotent stem cells has numerous ethical and practical problems. In order to produce patient-specific CMs, the application of somatic stem cells as an initial source is an appealing possibility. Somatic stem cells reside in almost all tissues of the human body. Pioneering experiments described cardiac differentiation from various somatic stem cells, including bone marrow cells, endothelial progenitors, and the widely used mesenchymal stem cells (MSCs). In the following section, we focus on cardiac cell generation from these latter cell types.

Human MSCs can be isolated from a variety of human tissues, including the adipose tissue, bone marrow, umbilical cord, and the amnion. Although all types of MSCs display similar morphology, surface marker pattern, immunogenicity, and differentiation potential toward osteogenic, adipogenic, and chondrogenic lineages (Lian *et al.*, 2007; Trivedi and Hematti, 2008), certain differences have been observed depending on the tissue source and *in vitro* culture conditions (Kern *et al.*, 2006).

According to several early reports, coculturing of MSCs with appropriate cardiac cells (CMs or cardiac fibroblasts) is an effective way for cardiomyocyte generation. In these reports, MSCs have been suggested to transdifferentiate into CMs (Li *et al.*, 2007; Xu *et al.*, 2004). When human MSCs were cocultured with neonatal rat ventricular CMs, MSCs differentiated toward a myogenic phenotype *in vitro* (Li *et al.*, 2007). Labovsky *et al.* (2010)

reported the cardiomyogenic differentiation of human bone-marrow-derived mesenchymal cells with a potential reprogramming of the hMSCs by a neonatal rat cardiomyocyte extract, and by using streptolysin O treatment.

Another way to generate human CMs is to treat MSCs with various chemicals or by activation of endogenous morphogenes. Antonitsis *et al.* (2008) reported that human adult bone marrow mesenchymal stem cells (BM-MSCs) from patients undergoing open heart surgery could be differentiated into CMs by an *in vitro* treatment with 5-azacytidine. These cultured MSCs connected with adjoining cells to form myotube-like structures and expressed cardiomyocyte-specific marker genes (Antonitsis *et al.*, 2008). It was also shown that the combination of Wnt11 and BMP-2 treatment in a temporal manner enhanced cardiomyogenic differentiation of BM-MSC, resulting in significant upregulation of cardiac-specific genes (Zhang *et al.*, 2012).

Most probably, the differentiation potential of human MSCs strongly depends on the source of these cells. Recently, Ramkisoensing *et al.* (2011) examined how human MSCs obtained from tissues in different developmental stages differentiated toward CMs, endothelial cells, or smooth muscle cells (SMCs). Rat neonatal CMs or cardiac fibroblasts were used as feeder cells for human MSCs, obtained from embryonic, fetal, or adult tissues. The properties of the resulting cardiac-like cells were assessed by α -actinin immunostaining, and the expression of human cardiac-specific genes (*NKX2-5*, *GATA-4*, *ANP*, *MLC-2V*, and *CX43*) were analyzed by qRT-PCR (quantitative real-time polymerase chain reaction). According to this study, the differentiation potential of hMSCs toward the three cardiac cell lineages depended on the developmental stage of the donor tissues. Human MSCs obtained from an embryonic or fetal source differentiated into the cardiac lineage much better than MSCs from adult tissues, and the gene expression profile and electrophysiological properties of these cells were similar to CMs (Ramkisoensing *et al.*, 2011).

These results are in line with earlier experimental results showing that neonatal tissues are the best potential sources of MSCs to be differentiated into CMs. Extraembryonic mesodermal cells obtained from the human placenta were able to differentiate into CMs and express cardiac-specific genes (Okamoto *et al.*, 2007). In addition, when MSCs

were isolated from the endothelial/subendothelial layers of the veins of the human umbilical cord, MSCs were transformed into cardiomyocyte-like cells upon 5-azacitidine treatment and expressed cardiac-specific genes (Kadivar *et al.*, 2006). Freshly isolated human amniotic mesenchymal cells (hAMCs) were found to express some cardiac-specific genes, and when stimulated with basic fibroblast growth factor (bFGF) or activin A, they expressed numerous cardiac-specific markers and differentiated into cardiomyocyte-like cells (Zhao *et al.*, 2005). The selection of cardiac progenitor cells based on a lentiviral gene expression was advocated by Bai *et al.* (2007). According to this report, cardiac progenitor cells could be isolated and enriched from human adipose-derived stem cells by using lentiviral expression of fluorescent proteins driven by cardiac-specific promoters.

MSC-derived CMs, according to recent studies, show increased expression of cardiac-specific genes (such as *NKX2-5* and *GATA4*) and perform important ultrastructural changes; however, their differentiation is still inefficient and incomplete (Arminan *et al.*, 2010; Mazo *et al.*, 2012). Although the exact determination or purification of the MSCs with high cardiogenic potential is to be established, these results indicate that human MSCs provide a potential source for CMs, both for drug screening and for therapeutic applications.

3.3 Direct Reprogramming of Mature Human Cells into Cardiomyocytes

For a long time, cell differentiation has been considered as a unidirectional process. Normally, cell fate decision is progressive and associates with a restricted differentiation potential and with increasing specialization. However, first by the method of “somatic cell nuclear transfer” (SCNT or cloning) (Campbell *et al.*, 1996) starting in the 1950s and then by the generation of iPSCs (Takahashi and Yamanaka, 2006), we have learned that differentiated adult somatic cells can return to an undifferentiated state, similar to that present in the embryo.

Reprogramming of adult cells to embryonic type cells paved the way for a new technique called *direct reprogramming*. Direct reprogramming occurs when a forced expression of lineage-specific transcription factors direct the cell fate from one mature somatic

cell type into a different, but similar mature somatic cell type, without the involvement of a pluripotent intermediate stage (Cohen and Melton, 2011; Graf, 2011; Vierbuchen and Wernig, 2011, 2012).

Direct reprogramming approaches offer exciting prospects for the future and may be suitable to provide large amounts of cells required for individualized drug screening or disease modeling. Moreover, a direct reprogramming technique could potentially avoid the danger of residual pluripotent cells, thereby eliminating the risk of teratoma formation during therapeutic approaches. Regarding CMs, direct reprogramming by various combinations of transcription factors have already been described. Ieda *et al.* (2010) first reported the direct *in vitro* reprogramming of adult mouse fibroblasts into CMs by forced retroviral expression of three cardiac transcription factors, *Gata4*, *Mef2c*, and *Tbx5*. These factors were selected from an initial pool of 14 key genes, playing essential role in cardiac development and associated with developmental cardiac defects in mutants. These induced cardiomyocytes (iCMs) exhibited spontaneous contraction activity and their Ca^{2+} oscillation frequency and AP resembled that of ventricular CMs. The global gene expression profile in the generated iCMs was reminiscent of the gene expression pattern shown by neonatal CMs. Two years later, the group of Srivastava confirmed the direct reprogramming strategy under *in vivo* conditions as well, when murine cardiac fibroblasts were transformed into functional CMs by using the same three transcription factors (Qian *et al.*, 2012).

Recently, Song *et al.* (2012) observed better *in vitro* direct reprogramming efficiency of mouse fibroblasts to CMs by adding a fourth transcription factor, namely *HAND2*, to the reprogramming cocktail. Similarly to Qian *et al.*, the authors induced myocardial infarction by coronary artery ligation in the mouse heart and used retroviruses to inject the transcription factor genes into fibroblasts along the border zone of the infarct.

Both research groups concluded that mouse cardiac fibroblasts can be more efficiently reprogrammed in their native environment, than *in vitro*, presumably due to the factors present within the native milieu of the intact heart. These factors may include extracellular-matrix-secreted proteins, growth factors, as well as direct interactions with the surrounding cells potentially promoting reprogramming. As a conclusion, induced mouse CMs were morphologically and functionally indistinguishable

from normal endogenous CMs and showed electrophysiological activities typical of this cell type, including AP and electrical coupling. Moreover, reprogramming after myocardial infarction reduced fibrosis and improved cardiac function.

In similar studies, numerous additional methods have been developed for the direct reprogramming of mouse cardiac cells. Efe *et al.* (2011) directed partially reprogrammed fibroblasts toward iCMs without going through a pluripotent intermediate state, by the additional application of an inhibitor of the JAK-STAT (Janus kinase-signal transducer and activator of transcription) signaling pathway. The protocol described by Efe *et al.* proved to be nearly three times as fast as transdifferentiation by overexpression of lineage-specific factors. An alternative strategy for the conversion of mouse fibroblasts to CMs by a nontranscription-factor-mediated method was reported by Jayawardena *et al.* (2012), when a combination of miRNAs were described to induce direct cellular reprogramming of cardiac fibroblasts into a cardiomyocyte-like phenotype, both *in vitro* and *in vivo*. This report has showed that mouse fibroblasts can be directly converted to CMs via a nonviral, lipid-based transfection method. Moreover, the miRNA-mediated reprogramming could be further enhanced with the JAK inhibitor treatment. Reprogrammed cells expressed cardiomyocyte genes and proteins, showed sarcomeric organization, and exhibited spontaneous calcium oscillations and contractility. On the basis of these findings, miRNAs seem to play a key role in cardiac reprogramming and may provide powerful tools for CM reprogramming, without the application of transcription factors.

Despite these detailed studies in mice, only one recent study documenting a potential direct conversion of human fibroblasts to fully differentiated and functional human CMs has been published. Islas *et al.* (2012) reported a successful direct conversion of normal human dermal fibroblasts (NHDFs) to the cardiac lineage through a lentivirus-mediated forced expression of the mammalian v-ets erythroblastosis virus E26 oncogene homolog 2 (*ETS2*) and the mesoderm posterior homolog (*MESPI*). Cardiac progenitors could also be generated by the introduction of purified fusion proteins containing *ETS2* and *MESPI* fused to a cell-penetrating peptide.

According to this study, *ETS2* overexpression activated the activin/nodal pathway in NHDFs but

blocked the appearance of smooth muscle genes, most likely through the inhibition of the BMP-Smad signaling pathway. Upon co-expression with *ETS2*, *MESPI* switched on the cardiac-specified gene program, in concert with activin A and BMP2, thereby reprogramming fibroblasts directly into cardiac progenitors. It should be mentioned that although a considerable number of NHDFs was successfully reprogrammed in this study, the emerging *ETS2/MESPI* transdifferentiated cells were not similar to mature CMs and therefore not directly suitable for drug discovery or cardiotoxicity applications.

Hopefully, successful methods for the generation of functionally mature iCMs through direct conversion of human cells will soon be established. These methods may be quickly adopted to generate disease-specific CMs for drug discovery and disease modeling, without the additional time and costs of generating disease-specific iPS cells and then differentiating them toward the cardiac lineage.

4 HUMAN PLURIPOTENT STEM-CELL-DERIVED CARDIAC PROGENITOR CELLS FOR LARGE SCALE PRODUCTION

Cardiac cells have to be produced in large numbers to satisfy the demands of drug testing applications, especially when high-throughput procedures are to be used. Generation of cardiac progenitors from pluripotent stem cells and then further enrichment and differentiation of the progenitor cells may provide suitable preparations for these demands.

As CMs undergo progressive cell cycle withdrawal during maturation (Snir *et al.*, 2003), the early steps of the differentiation process has to be optimized to enhance cardiomyocyte yield. To this end, numerous *in vitro* differentiation protocols have been developed, attempting to recapitulate the stepwise stages of cardiogenesis in the embryo (Laflamme *et al.*, 2007; Yang *et al.*, 2008). A major drawback is the variable efficiency of the differentiation protocols across different hPSC lines, which has not been fully eliminated yet (Sepac *et al.*, 2012), even though an universal and highly efficient cardiac differentiation system for hPSC has been recently introduced (Burrige *et al.*, 2011), which may revolutionize the field.

To achieve a major enhancement of CM yield, an alternative or complementary strategy to the optimization of cardiac differentiation protocols would be to suspend the differentiation process at a certain stage, when the cells still possess high proliferation capacity, but are more or less restricted in their differentiation potency. After expanding these specific progenitor cells, CM maturation should be achieved.

Recently, hESC-derived definitive endoderm and endocrine progenitors, representing different stages of development within the pancreatic lineage, were used to demonstrate that progenitor cells are able to self-renew and proliferate without differentiation when cocultured with organ-matched primary mesenchymal cell lines (Sneddon, Borowiak, and Melton, 2012). In this process, lineage- and stage-specific renewal signals may replace the need for supporting primary cell lines. Although cardiac progenitors were shown to be able to proliferate (Bu *et al.*, 2009), proper culture conditions supporting continuous self-renewal and proliferation have not been described yet.

For an efficient technology, before propagation, progenitors have to be isolated to avoid further differentiation due to factors secreted by other differentiating cells in their microenvironment. Therefore, the discovery of markers specifically identifying progenitor cells at different stages of the cardiac differentiation is an important task. In the following section, recent achievements in the identification of cell surface markers and the establishment of genetically modified features, enabling the isolation of progenitor cells with cardiomyogenic potential, are introduced. Marker characteristic for specific stages of the differentiation is discussed in the order outlined by the hierarchy of cardiac development, from primitive streak-like/nascent mesoderm progenitor cells, through multipotent cardiovascular progenitors, to differentiated CMs (Figure 2).

During hESC differentiation, the first defined progenitor cells that are able to generate CMs are the primitive streak-like cells, expressing specific transcription factors, for example, the T-box factor *BRACHYURY*(T) and the homeodomain protein *MIXL1*. Primitive streak-like cells are *mesendodermal progenitors* as they are able to give rise to all mesodermal and endodermal lineages. In the presence of adequate concentrations of mesoderm-inducing agents, such as BMP4 and activin A, these cells differentiate mostly into the

mesodermal lineage and become *mesodermal progenitors*. Mesodermal progenitors are able to give rise to the hematopoietic, vascular, cardiac, and skeletal muscle lineages.

A potential method to isolate these progenitors is based on the use of genetically modified reporter hESC lines, expressing fluorescent proteins driven by selected promoters. Genetically modified features are specially required when the known markers of a progenitor stage are not located on the cell surface (e.g., transcription factors or sarcomeric proteins) and therefore selection of the progenitors would not be possible without disruption of the cell membrane. An example of this technique was reported when mesodermal precursors expressing the transcription factor *BRACHYURY* could be isolated by using a randomly inserted *GFP* (*green fluorescent protein*) transgene under the control of the *BRACHYURY* promoter (Kita-Matsuo *et al.*, 2009). The expression of the *BRACHYURY*-promoter-driven GFP was maximally upregulated in the EBs between days 3 and 5.

A similar method for the isolation of early mesodermal precursors was established when sequences encoding GFP were targeted into the *MIXL1* locus by homologous recombination, in order to allow a selective isolation of *MIXL1* expressing cells (Davis *et al.*, 2008). Upregulation of *MIXL1*-GFP was dependent on the presence of BMP4 and regularly started on day 3 of differentiation. GFP expression peaked in EBs either at days 4–6 or at day 7, and lasted until day 10 or 12, depending on the growth factor cocktail composition applied to promote differentiation. *MIXL1*-GFP positive cells initially expressed E-cadherin and 50% of them were platelet-derived growth factor receptor- α (PDGFRA) positive as well. E-cadherin positivity gradually decreased during further differentiation, suggesting that the cells had undergone epithelial-to-mesenchymal (EMT) transition. EMT can be considered as an early marker of differentiation, as mesodermal commitment from hESCs was shown to be initiated by EMT (Evseenko *et al.*, 2010). Parallel to the gradual loss of E-cadherin positivity, the proportion of PDGFRA positive cells within *MIXL1*-GFP⁺ cells continuously increased, until the downregulation of *MIXL1* occurred at about day 12.

It was shown, that at a specific stage of differentiation, mesodermal progenitors upregulate the tyrosine kinase transmembrane receptor (ROR2)

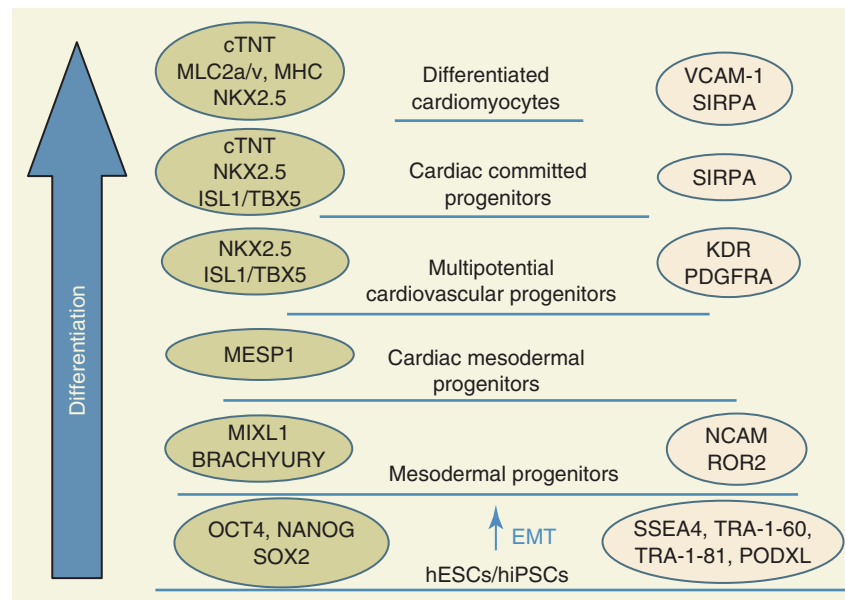


Figure 2. Proposed progenitor stages of the differentiation of human embryonic stem cells (hESCs) and induced pluripotent stem cells (hiPSCs) into differentiated cardiomyocytes identified by transcription factors and structure proteins (shown in green bubbles) and cell surface markers (shown in gray bubbles).

(Drukker *et al.*, 2012), which allows the isolation of these cells without the need of genetic manipulation. Isolated $ROR2^{+}$ cells expressed moderate to high levels of mRNA transcripts of genes known to be involved in mesoderm formation, including *BRACHYURY*, *MESP1*, and *MIXL1*. Strong downregulation of E-cadherin and upregulation of N-cadherin could be observed when the isolated $ROR2^{+}$ cells were recultured for 1 week, demonstrating that these cells had undergone EMT during the culture. At this time point, upregulation of several genes, such as *BRACHYURY*, *MESP1*, and *ISL1* (LIM homeodomain transcription factor), and surface markers, such as kinase insert domain receptor (KDR) and PDGFRA, confirmed the mesoderm commitment of $ROR2^{+}$ cells. The authors suggested that $ROR2^{+}$ cells are similar to early primitive-streak precursors undergoing EMT and lateral mesoderm induction during migration in the mouse embryo (Arnold and Robertson, 2009).

Mesodermal progenitors, derived from hESCs via EMT, were shown to express the neuronal cell adhesion molecule (NCAM or CD56) but not the epithelial cell adhesion molecule (EpCAM or CD326) (Evseenko *et al.*, 2010). This $CD326^{-}/CD56^{+}$ population became clearly detectable at day 3.5 of

differentiation in a mesoendoderm-inducing condition. Microarray analysis of $CD326^{-}/CD56^{+}$ cells isolated at day 3.5 revealed a mesodermal-specific gene expression pattern; among several other genes, *BRACHYURY* and *MESP1* were also upregulated. At this time point, KDR was quite downregulated and the expression of PDGFRA was not detectable at all. By day 5, subsets of the $CD326^{-}/CD56^{+}$ population began to express KDR, CD34, and PDGFRA. All mesodermal lineages, including hematopoietic, endothelial, mesenchymal (bone, cartilage, fat, and fibroblast), smooth muscle, and CM, could be generated from $CD326^{-}/CD56^{+}$ cells isolated at day 3.5, while these cells failed to give rise to ectodermal or endodermal cell types. Approximately 5–10% of the generated cells were CMs. However, mention must be made, that previously, this subset of markers was documented to identify hESC-derived neuronal cells, as more than 80% of these cells expressed CD56 and at the same time most of them were CD326 negative (Sundberg *et al.*, 2009).

Activation of the basic helix–loop–helix transcription factor, mesoderm posterior 1 (*MESP1*), indicates the onset of cardiac mesoderm formation *in vivo*. It was shown that induction of *MESP1* by

BRACHYURY generates the common multipotent cardiovascular progenitor from mouse ESCs (David *et al.*, 2011). The mature heart is composed of multiple cell types, including CMs, endothelial cells, and SMCs, all arising from two types of multipotent cardiovascular progenitors, the primary and the secondary heart fields. *MESPI* expressing mESC-derived cells was shown to represent a common progenitor for the multipotent cardiovascular progenitors of both heart fields (Bondue *et al.*, 2008).

ISL1 is a marker of the secondary heart field. Isolation and lineage tracing of ISL1⁺ early multipotent cardiovascular progenitors became possible through the use of a *Cre/loxP* system; first human embryonic *ISL1-cre* cells were generated with a *cre-IRES-puro* cassette targeted into the *ISL1* locus by homologous recombination, then pCAG-flox-DsRed plasmids were transfected into *ISL1-cre* hESCs, and finally cre expression-mediated excision of the floxed stop sequence on the pCAG-flox-DsRed plasmid resulted in irreversible expression of the DsRed gene in ISL1⁺ cells and in their progeny (Bu *et al.*, 2009). DsRed⁺ cells could be first detected at days 5–6, two days before the upregulation of *NKX2-5*, and when isolated at EB day 8, DsRed⁺ cells did not express KDR as well. Nevertheless, ISL1⁺ cells were able to give rise to the three major cardiac lineages, with a CM yield of ~4%. A limited proliferation capacity could be observed when ISL1⁺ cardiovascular progenitors were cocultured with a Wnt3A-secreting feeder instead of MEF.

The stage-specific embryonic antigen-1 (SSEA-1) has also been proposed to be suitable for the isolation of multipotent cardiovascular progenitors, as isolated SSEA-1⁺ cells were shown to express cardiac lineage-specific markers, including *MESPI*, *MEF2C*, *NKX2.5*, *GATA4*, *ISL1*, and *TBX5*, a marker of the primary heart field (Blin *et al.*, 2010). However, it was previously reported that SSEA-1 is expressed on hESC-derived epithelial (Thomson *et al.*, 1998) or neuroectodermal committed cell types (Draper *et al.*, 2002), thus SSEA-1-based selection may only allow enrichment of multipotent cardiovascular progenitors when the differentiation has already been efficiently directed into the cardiac lineage.

At EB day 6 of cardiac-lineage-directed differentiation, a population of cardiovascular progenitor cells expressing *ISL1* and low levels of *NKX2-5* and *TBX5* could be isolated, based on the low

expression levels of the KDR, also called *vascular endothelial growth factor receptor 2* (*VEGFR-2*), and on the absence of the mast/stem cell growth factor receptor, also known as *proto-oncogene c-Kit* or *tyrosine kinase kit* (*C-KIT* or *CD117*) (Yang *et al.*, 2008). This KDR^{low}/C-KIT^{neg} population displayed cardiac, endothelial, and vascular smooth muscle potential, with a CM yield of 40%, when cultured as aggregates, or more than 50%, when plated as monolayers. Higher CM yield (up to 80%) could be achieved when isolation was based on the co-expression of KDR and PDGFRA at day 5 of differentiation (Kattman *et al.*, 2011). Similarly, high CM yield could be achieved without isolation of the KDR⁺/PDGFRA⁺ cells, when activin A and BMP4 concentrations were optimized to highly enrich these double positive cells in the culture. However, as KDR is also expressed on the cell surface of undifferentiated hPSCs, an isolation procedure based on this marker may result in an impure population of cardiac progenitors.

Multipotential cardiovascular progenitors could be isolated when *eGFP* was introduced into the *NKX2-5* locus of a hESC line by homologous recombination and the NKX2-5^{eGFP/w} hESC line was differentiated as spin EBs or by coculture with the END2 cell line into the cardiac lineage (Elliott *et al.*, 2011). This reporter system was utilized in various ways; first, the GFP signal was used to optimize the concentrations of BMP4 and activin A in the culture medium to enhance the efficiency of cardiogenesis. Stage-specific reporter systems generally allow detecting the result of the optimization at specific stages of the differentiation, which provides a more direct way of monitoring than relying on the final CM outcome. Another advantage of the fluorescent reporter cell line was utilized when two new cell surface markers of the cardiac lineage could be identified based on the gene expression profiling of isolated eGFP⁺ cells, namely vascular cell adhesion molecule 1 (VCAM-1) and signal regulatory protein alpha (SIRPA). Further experiments showed that VCAM-1 positivity appears slightly later than SIRPA (Elliott *et al.*, 2011).

SIRPA is expressed on *cardiac-committed progenitors* and *differentiated CMs* but not on the KDR⁺/PDGFRA⁺ population of cardiac mesoderm cells as reported by another research group using the same reporter cell line (Dubois *et al.*, 2011). In this experimental setup, when differentiation was carried out through EB formation, the first

NKX2-5-GFP⁺ cells developed at EB days 7–8 parallel to the upregulation of SIRPA. Contracting CMs were first detected between days 9 and 12; thus, SIRPA⁺ cells isolated at EB day 8 were considered as cardiac precursors and were able to give rise to troponin-positive CMs.

VCAM1-expression-based efficient purification of hPSCs-CMs was reported by the research group of Yamashita as well (Uosaki *et al.*, 2011). They showed that troponin expression precede VCAM1 appearance; thus, VCAM1 allows the isolation of early CMs, but not progenitor cells. VCAM1-expression-based isolation resulted in highly purified (>95%) troponin-T-positive CMs in the case of all four hPSC lines examined, although the VCAM1-positive cells represented only a subset within troponin-T-positive cells, which might reflect differences in maturation status among troponin-T-positive cells.

Early CMs represent a less feasible source for upscaling, but markers identifying more differentiated CMs enable purification of the upscaled population of isolated multipotent progenitors, and thus the generation of pure CM populations can be achieved. However, it is important to keep in mind that the significance of markers identifying a specific progenitor population may be specific to the culture condition, as not only progenitors of the cardiac lineage and CMs may express these markers, when cells of all three germ layers and with different maturation statuses are present.

5 REPORTER SYSTEMS USED TO FOLLOW CARDIAC DIFFERENTIATION

There are several ways to generate human CMs using different cell types, as it has been discussed in the previous sections in detail. However, for drug screening, transplantation studies, or basic research, we need a large number of pure CMs. Irrespective of cell types and differentiation protocols, we have to *mark* the cells, *follow* the differentiation process, *select* the progenitor or mature cardiac cells, and perform *functional characterization* before further applications. Fluorescent proteins, for example, different versions of the GFP and several red fluorescence markers including mCherry, tomato or dsRed proteins, or the yellow fluorescent protein (YFP), are the most commonly used markers for such purposes. In addition, luminescence reporters,

for example, firefly or renilla luciferase (LUC), or the LacZ protein are widely used for tracking cells especially in animal studies. Prelabeling of the transplanted cells with fluorescent cell tracers, such as vybrant-carboxyfluorescein diacetate (CFDA) and succinimidyl ester, can also be applied (Caspi *et al.*, 2007). For selection or enrichment of the desired cell population, several methods have been developed, ranging from manual dissection of the beating areas (Huber *et al.*, 2007; Mummery, Ward, and Passier, 2007) to various biochemical and elaborated genetic screening methodologies (Anderson *et al.*, 2007; Huber *et al.*, 2007; Kita-Matsuo *et al.*, 2009; Klug *et al.*, 1996; Laflamme *et al.*, 2007; Muller *et al.*, 2000). Other techniques are also applied to characterize the physiological status of the selected cells, including electrophysiological measurements of action potentials (He *et al.*, 2003; Jonsson *et al.*, 2010) or the examination of other functional features of selected CMs (Chen, Kim, and Mercola, 2009; Dolnikov *et al.*, 2006; Kehat *et al.*, 2001; Kita-Matsuo *et al.*, 2009; Liu *et al.*, 2007; Mandel *et al.*, 2012).

The use of genetically engineered reporter systems can help overcome the above-mentioned problems. Generation of reporter-expressing CMs differentiated from stem cells raises new technical challenges; genetic modification of stem cells is less efficient than that of tumor-derived or immortalized cell lines (Section 2). Moreover, the low efficiency of cardiac differentiation protocols often requires further selection procedures to generate pure or at least enriched populations of transgene-expressing CM. Constitutive promoters (such as EF1 α , PGK, CAG, or CMV) provide uniform transgene expression in both the undifferentiated and the differentiated forms of stem cells. This approach is applicable for marking the transgene-positive stem cell population after gene delivery and is suitable for tracking transgene expressing cells in animal models. This approach was applied for studying the improvement of postinfarcted hearts after transplantation of ESC-differentiated cardiac cells in rat and mouse models. Using the CMV-driven GFP transgene to follow the transplanted cells, improved cardiac function after transplantation was demonstrated in both cases (Min *et al.*, 2002; Yang *et al.*, 2002). Similar strategies were applied using human ESC-derived CMs in infarcted rat heart (Caspi *et al.*, 2007) or when CMs differentiated from GFP expressing HUES3 cells survived and further matured in rat

and mouse hearts, causing transient improvements after myocardial infarction (Dai *et al.*, 2007; van Laake *et al.*, 2007). When Xue *et al.* (2005) studied the integration of hESC-derived CMs into host cardiac tissues both *in vitro* and *in vivo*, a CAG-promoter-driven GFP expression provided the basis of identification of transplanted cells. These cells functionally integrated together with isolated rat ventricular CMs and induced electrical and contractile activities; moreover, they modified the excitability of the guinea pig heart after transplantation *in vivo*.

Improvement of gene delivery systems and cardiac differentiation protocols allowed the use of cardiac-tissue-specific promoters, although in these cases, transgene activity can only be detected at a certain stage of differentiation. For selection of cardiac progenitor cells, promoters of early cardiac-commitment-specific genes, such as *NKX2-5* or *BRACHYURY*, were identified, while for selection of more matured CMs, promoters of myosin light chain-2v (*MLC-2V*), atrial natriuretic peptide (*ANP*), cardiac troponin-I (*TNNI3*), *alpha myosin heavy chain* (*αMHC*), or the striated-muscle-specific *cytokeratine 7* (*CK7*) genes were chosen. When, for example, the *MLC-2V* promoter was used, a transgene-positive population appeared without pacemaker activity, which could further progress toward cells with a ventricular-like cardiac phenotype (Muller *et al.*, 2000). The *ANP* promoter (Gassanov *et al.*, 2004) or the human ventricular *MLC-2* promoter (Huber *et al.*, 2007) could be used to generate pacemaker-cell-rich populations, and in the latter case, the mechanically dissected GFP-positive CMs expressed cardiac-specific proteins, displayed AP, and formed stable myocardial cell grafts following *in vivo* cell transplantation into a rat model. Recently, *αMHC*-promoter-driven cardiomyocyte selection has been suggested to provide an appropriate mean to establish cardiac precursor populations, which can later give rise to multiple CM subtypes for cell therapy and drug screening studies in various human and animal model systems (Bailey *et al.*, 2009; Ieda *et al.*, 2010; Otsuji *et al.*, 2010; Qian *et al.*, 2012; Ritner *et al.*, 2011; Song *et al.*, 2012).

Gallo *et al.* (2008) suggested that a short proximal fragment of the cardiac troponin-I proximal promoter is sufficient to confer cardiac-specific gene expression in both human and mouse cells. Using a third-generation lentiviral system, the 340-bp

fragment of the *hTNNI3-50* flanking region was cloned upstream of the reporter gene and used to transduce mouse and hESCs; cardiac differentiation was carried out via the EB or the END-2 coculture system. Owing to a certain degree of nonspecific expression in undifferentiated cells, these authors further increased cardiac specificity by combining the *TNNI3* promoter with the human cardiac *α-actin* enhancer. These results indicated that this arrangement is suitable for marking the cardiomyogenic lineage in human and mouse ESC differentiation systems.

Application of drug resistance genes is another possibility for selection of targeted cell population. *αMHC*-promoter-driven puromycin resistance gene was used for positive selection of hESC-derived CMs with relatively high (80–90%) efficacy (Anderson *et al.*, 2007; Xu *et al.*, 2008a). Taking the advantage of the quiescent feature of CMs, Anderson *et al.* (2007) applied herpes simplex virus thymidine kinase/ganciclovir (PGK-HSVtk/GCV) suicide gene insertion system for negative selection of proliferating cells and obtained more than 30% purity for CMs. Neither positive nor negative selection caused functional alterations of functionality of the differentiated CMs.

Another efficient approach is to apply a double positive selection strategy. In such cases, stable transgene expressing ESC lines can be established first, which provide the potential to select CMs based on the appearance of another expressed transgene in later steps of differentiation. The increased size of the vector constructs may lower gene delivery efficiency; nevertheless, such platforms seem popular to generate CMs. As an example, GFP expression can be driven by the constitutive *EF1α* promoter to select successfully transduced undifferentiated hES cells, while an additional *MLC2V*-promoter-driven *dsRed* expression was applied for mechanical enrichment and further purification of differentiated CMs. CMs obtained by the latter approach showed ventricular AP and were used for the characterization of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger protein (Fu *et al.*, 2010). Various promoters, specific for either the undifferentiated stage or the particular cardiac cell types, are generally used in combination to express fluorescent transgenes and/or transcription units conferring drug resistance. In addition, recently developed genome editing technologies (described in Section 2) could also be used to insert transgenes downstream of endogenous cardiac-specific gene

promoters, thereby allowing the lineage tracing of cardiac-specific cell types (Bu *et al.*, 2009; Fehling *et al.*, 2003; Gantz *et al.*, 2012; Hidaka *et al.*, 2003). However, a major concern is that the integration of these transgenes or the selection procedures *per se* may alter the physiological characteristics of the obtained cell populations. These problems further strengthened the need for functional/physiological testing of the generated cells. Still, in many cases, the studies reassuringly suggested that the obtained CMs were physiologically normal, regardless of the isolation procedure (Kita-Matsuo *et al.*, 2009).

Another recent approach is to use a “double-feature” promoter, instead of the double promoter systems described earlier. Orban *et al.* found that a certain version of the widely used CAG promoter is suitable to mark genetically modified undifferentiated hES cells. At the same time, this approach also provided a selection platform specific for CMs during subsequent spontaneous differentiation. This version of CAG promoter was named as “double-feature” promoter, as apart from being constitutively functional in all cell types (including undifferentiated ESCs), its transcriptional activity became exceptionally high specifically in cardiac tissues. By utilizing these characteristics, stable GFP-expressing hESC clones were established by a transposon-based gene delivery method, and in a further differentiation stage, CMs could be sorted on the basis of the extremely high GFP expression (Orban *et al.*, 2009). Another potential application of this “double-feature” promoter was documented recently, when cardiomyocyte functions were studied by using a genetically encoded calcium indicator (GECI) system (Whitaker, 2010), providing unique opportunities to investigate intracellular calcium signals in neural (Zariwala *et al.*, 2012) or in cardiac tissues (Tallini *et al.*, 2006).

In these studies, a “double-feature” CAG-promoter-driven GCaMP2 calcium indicator gene construct was stably introduced into hESCs by using the SB transposon-based gene delivery system. The GCaMP2 expression provided an excellent platform to measure changes in intracellular calcium levels both in hESCs and hESC-derived cardiac (Supplementary video 1) and neural cells (Apati *et al.*, 2013). A similar reporter system was applied when the ZFN technique was used for the expression of the GCaMP3 calcium indicator inserted into the endogenous AAVS1 locus of the hESCs. The GCaMP3-expressing hESC-derived CMs were

transplanted into injured guinea pig hearts in order to study *in vivo* supporting effects of transplanted human cells (Shiba *et al.*, 2012).

As a summary of the above-described approaches, currently, there are three promising strategies for selecting and labeling human pluripotent stem-cell-derived CMs with genetically engineered reporter systems. Constitutive promoters can be used for checking the efficiency of gene delivery and for selection of transgene expressing stem cell clones, but these methods are inappropriate for selection of stem-cell-derived CMs. Cardiac-specific promoters are suitable for the identification and the assortment of cardiac progenitor or cardiac type cells, though highly efficient gene delivery methods and differentiation protocols are essential in this case. Combination of constitutive and tissue-specific promoters and usage of artificial “double-feature” promoters are novel solutions for the isolation of ESC-derived reporter-gene-expressing CMs with high efficiency. By using these technologies, CMs can reliably be obtained for further electrophysiological, drug screening, or transplantation studies.

6 CARDIOTOXICITY OF DRUGS—KEY SCREENING SYSTEMS FOR DRUG DEVELOPMENT

Cardiotoxicity occurs when a compound has a harmful effect either on the musculature or on the electrophysiology of the heart. In severe cases, the consequence of cardiotoxicity may lead to a life-threatening heart failure. Cardiotoxic compounds may damage CMs through various mechanisms, including inflammation, apoptosis, mitochondrial dysfunction, DNA damage, or generation of reactive oxygen species. Compounds interacting with ion channels or membrane transporter proteins impair heart function through alteration of the electrophysiological properties of myocytes, which may lead to a pro-arrhythmic condition and later to ventricular arrhythmias.

Many drugs had to be recalled from the market due to cardiotoxic side effects, in some cases, resulting in a dangerous form of ventricular arrhythmia, called *Torsade de pointes* (TdP). As an example, terfenadine, an efficient antihistaminic agent, was withdrawn from the market because of arrhythmic side effects, and several antibiotics, antipsychotic, and cardiovascular drugs are also known to induce

TdP (www.azcert.org). In addition, many other drugs carry a warning sign about potential cardiac side effects, required by the Food and Drug Administration (FDA).

Prolongation of the QT interval, the time between two electric peaks representing the beginning of the ventricular depolarization and the end of the ventricular repolarization on an electrocardiogram, is considered to increase the risk of TdP (Haverkamp *et al.*, 2000). The long QT (LQT) syndrome may be inherited or acquired, and both forms are characterized by the abnormal activity of specific ion channels. The most common causes of acquired long QT syndrome are drugs, but cardiac disorders alone or in combination with drugs can also lead to this condition. Thus drug developers had to address the question of the “LQT” phenomenon for all drugs seeking general approval, and assessing drug-induced QT prolongation has become one of the most frequently used biomarkers in cardiac safety tests. However, 89% of the drugs tested by preclinical cardiac safety tests still proved to be toxic or ineffective in the clinical phase of the drug development process (Kaitin, 2008; Kola and Landis, 2004; Sollano *et al.*, 2008). The high amount of failed compounds indicates the inadequacy of current *in vitro* and animal-model-based cardiac safety tests.

The mechanism underlying *drug-induced LQT* syndrome is almost always the blockade of ion channels encoded by the *KCNH2* (hERG) gene that provide the rapidly activating delayed rectifier potassium current I_{Kr} (Fenichel *et al.*, 2004; Haverkamp *et al.*, 2000; Roden *et al.*, 1988; Sanguinetti *et al.*, 1995; Sanguinetti and Jurkiewicz, 1990). In order to assess cardiotoxic drug effects on I_{Kr} , one solution is to use mammalian cell lines [e.g., human embryonic kidney cells (HEK293), mouse L cells, and Chinese hamster ovary (CHO) cells] ectopically expressing *KCNH2* ion channels (Lacerda *et al.*, 2008; Rodriguez-Menchaca *et al.*, 2008). However, these systems provide only limited information on the actual effect of the tested compound on the human heart because of the genetic aberrations present in these cells and the lack of other voltage-gated channels characteristic to human CMs (Liang *et al.*, 2013; Lu *et al.*, 2008).

Other commonly utilized *in vitro* test systems for cardiotoxicity screens rely on multicellular preparations, such as the dog or rabbit Purkinje fibers, the ventricular wedge preparation, or the Langendorff

heart preparation (Kannankeril, Roden, and Darbar, 2010). Commonly used *in vivo* animal models in preclinical cardiotoxicity assays include multi-lead ECG recordings in conscious or anaesthetized guinea pigs, rabbits, dogs, or pigs (Haverkamp *et al.*, 2000). However, both *in vitro* and *in vivo* animal models are in many cases inadequate because of the major interspecies differences (Akar *et al.*, 2004; Blechschmidt *et al.*, 2008; Jung *et al.*, 2012a; Rosati *et al.*, 2008; Zicha *et al.*, 2003). The costs of purchasing and maintaining animal models as well as ethical issues concerning the use of these model systems are also commonly cited reasons for replacing or at least complementing animal models with human stem-cell-based *in vitro* systems.

Human cardiac tissues obtained from healthy donors have limited availability and, in addition, are heterogeneous and show high interbatch variability. The problems render difficult the use of human primary cultures of CM for wide-scale cardiotoxicity screenings. In addition, isolated human adult ventricular CM show a rapid loss of their sarcomere and myofibril structure in culture, which can be considered as de-differentiation (Li *et al.*, 1996). An alternative solution was reported by Smits, van Oorschot, and Goumans (2012), when isolation and subsequent expansion of human cardiomyocyte progenitor cells derived from cardiac surgical waste or fetal heart tissue were shown to be able to differentiate into CMs *in vitro*.

However, a consistent and renewable source of CMs can most probably be assured by using hPSC-derived CMs (Sections 3 and 4). In addition, patient-specific genetic polymorphisms, resulting in variable responses to administered drugs, cannot be estimated by using the currently available systems, whereas CMs derived from patient-specific hiPSC or directly reprogrammed from easily accessible patient-specific tissues advance the progress of personalized cardiotoxicity tests.

The effect of certain cardioactive drugs has already been tested on hPSC-derived CMs to validate these *in vitro* systems as reliable tools for human cardiotoxicity tests, with the ability to faithfully recapitulate the effect of the drug seen in patients (Braam *et al.*, 2010; Caspi *et al.*, 2009; He *et al.*, 2003; reviewed by Harding *et al.* (2007)). It has to be emphasized that human embryonic and induced pluripotent SC-derived CMs proved to be equally relevant models for normal heart physiology (Gai *et al.*, 2009; Tanaka *et al.*, 2009; Yokoo *et al.*, 2009).

In these studies, several platforms were used to detect changes in electrophysiological properties evoked by the administered drug. These platforms include traditional patch clamp (Gai *et al.*, 2009; Ma *et al.*, 2011; Sartiani *et al.*, 2007; Satin *et al.*, 2004; Tanaka *et al.*, 2009; Yokoo *et al.*, 2009) or different types of multielectrode arrays (MEA) [(Braam *et al.*, 2010; Caspi *et al.*, 2009; Tanaka *et al.*, 2009; Yokoo *et al.*, 2009)—reviewed by Asai *et al.* (2010)], detecting the surface electrogenic activities of human SC-derived CM clusters. As the patch clamp technique is limited to individual cells, MEA-based systems seem to be more suitable for high-throughput drug screens. An automated video-microscopy setup was used to analyze drug-induced changes in a 3D engineered heart tissue (EHT), generated from hESC-CMs (Schaaf *et al.*, 2011). 3D structures could also enable studies more relevant to clinical phenotypes of hypertrophic cardiomyopathy or arrhythmias.

However, some shortcomings of hPSC-CM-based test systems should be mentioned. The ideal cell preparation for accurate cardiotoxicity screenings would be a homogeneous population of hPSC-CMs, with a phenotype similar to adult human ventricular cells, as mainly the ventricular myocytes are affected by cardiotoxic side effects. Unfortunately, hPSC-CMs are generally a mixture of atrial, ventricular, and nodal cardiac cell types (He *et al.*, 2003; Ma *et al.*, 2011; Mummery *et al.*, 2003; Zhang *et al.*, 2009); therefore, the establishment of methods to obtain a relatively homogeneous population of ventricular myocytes (Zhang *et al.*, 2011; Zhu *et al.*, 2010) is an important task. Another shortcoming may be the potential immature, fetal-like phenotype of hPSC-CMs, manifesting in fetal CM-like organization of the sarcomeric proteins (Snir *et al.*, 2003), and electrophysiological properties (Dolnikov *et al.*, 2005; He *et al.*, 2003; Sartiani *et al.*, 2007; Satin *et al.*, 2004). On the other hand, the immature phenotype can become more mature during prolonged *in vitro* differentiation of hPSC-CMs (Lundy *et al.*, 2013), or through a forced expression of Kir2.1, encoding the inwardly rectifying K⁺ current (I_{K1}), which can render the immature electrophysiological phenotype of hPSC-CMs indistinguishable from the adult counterparts (Lieu *et al.*, 2013). Nevertheless, comparative studies have shown that pro-arrhythmic potential of standardized hESC-CMs are qualitatively and quantitatively

comparable to the rabbit Purkinje fiber model (Jonsson *et al.*, 2010). Moreover, hESC-derived CM clusters containing dominantly ventricular CMs showed greater pharmacological sensitivity than conventional rabbit or canine Purkinje fiber assays (Peng *et al.*, 2010).

All these approaches confirmed the applicability and the feasibility of human SC-derived CM systems, which enable the assessment of cardiotoxic side effects of novel drug candidates under biologically relevant conditions. Here, we again emphasize the power of tests based on disease-specific human iPSCs and their progenies, which may revolutionize not only the process of drug development but also the field of drug discovery. Therefore, we dedicated the next chapter to review already established model systems, allowing the assessment of specific disease conditions. These are based either on the reprogramming of diseased somatic cells to generate CMs, recapitulating faithfully the phenotype of the disease *in vitro*, or on the hPSC-derived CMs, as models to examine relevant parameters of cardiac physiology.

7 APPLICATION OF HUMAN CARDIOMYOCYTES FOR DISEASE MODELS AND PERSONALIZED DRUG DEVELOPMENT

In the last decades, several model systems have been used to investigate the physiology of the normal and diseased heart, including numerous animal models [(Chu, Haghighi, and Kranias, 2002; Nerbonne *et al.*, 2001), reviewed by Zaragoza *et al.* (2011)] and isolated primary cultures of nonhuman embryonic/neonatal (Bester and Gevers, 1975; DeHaan and Hirakow, 1972; Goshima and Tonomura, 1969; Harary and Farley, 1960; Kasten, 1972) or adult (Chen *et al.*, 2013; Ku, Chen, and Su, 2013; Louch *et al.*, 2004; Mitcheson, Hancox, and Levi, 1998; Piper, Jacobson, and Schwartz, 1988; Shahzad *et al.*, 2013) cardiac myocytes. *In vivo* studies mimic the events of heart injury but are less suitable for studying molecular mechanisms responsible for the development of this phenomenon. *In vitro* cell cultures of isolated primary CMs and cardiac progenitors could serve as models for studying homogenous cell populations, which are easy to visualize and manipulate, and the properties of these CMs can be examined in a controlled environment.

However, none of these models properly reflects any human disease because of significant interspecies differences. The use of human cardiac cells in disease modeling and drug screening applications may bridge the gap between the experimental results obtained from the animal models and the effects seen in humans.

Viable contracting CMs can be obtained from human atrial and ventricular tissues. However, adult CMs do not remain stable during long-term experiments; moreover, a limited availability of human heart tissue samples as well as the lot-to-lot variation renders these types of cardiac cells difficult to use.

Generation of hESC-derived CMs by several protocols (Section 3.1) presents another new cellular platform for the investigation of cardiotoxicity and cardiac diseases. The first disorder investigated with a hESC-CM-based model system was obstetric cholestasis (Abdul Kadir *et al.*, 2009), a rare complication of pregnancy, characterized by increased levels of serum bile acids, which can lead to unexplained fetal death at late gestation. The assumed pathomechanism behind fetal death was the enhanced arrhythmogenic effect of bile acids in the fetal heart. Immature hESC-CMs were shown to exhibit a bile-acid-induced disruption of rhythm, depression of contraction, and desynchronization of cell coupling, whereas mature CMs become resistant to these arrhythmias, thereby the assumed pathomechanism could be confirmed.

Foldes *et al.* (2011) showed that hESC-CM can be used as a hypertrophic model system for cardiac research and drug discovery/toxicology, as hESC-CMs retain their capacity for increase in size and volume in long-term culture.

Human ESC-based models of genetic disorders were generated by genetic modification of existing normal hESCs (Urbach, Schuldiner, and Benvenisty, 2004), although no data have been published focusing on genetically modified human ESC lines, modeling a cardiac disease. An alternative method to obtain hESC-based models of inherited monogenic diseases for drug screening and for the development of new treatments is based on the application of embryos with genetic disorders diagnosed in preimplantation genetic diagnosis (PGD) (Aran *et al.*, 2012; Frumkin *et al.*, 2010; Mateizel *et al.*, 2006; Taei *et al.*, 2010; Tropel *et al.*, 2010).

Currently, hiPSCs are the most promising cell source to study congenital heart failures “in a dish,” providing a defined genetic and environmental

background of diseases for these investigations. Disease-specific hiPSC lines can provide an almost unlimited source of diseased CMs, continuously providing the same quality and features. By using these cells, the phenotype of the disease and the molecular mechanisms hidden behind cardiac disorders can be studied, or drug candidates can be tested, aiming either to rescue the disease phenotype or to detect cardiotoxicity, even in high-throughput applications.

Antiarrhythmic drug treatments involve high incidence of serious side effects; hence, studies investigating the circumstances of cardiotoxicity play an important role in suitable pharmaceutical treatments of cardiac diseases. Drug-induced QT prolongation may not automatically lead to the development of pro-dysrhythmia (Fabritz and Kirchhof, 2010); therefore, some of the drugs known to induce QT prolongation would be still applicable in the treatment of diseases, if patient-specific screening assays could provide information about pro-dysrhythmic risks at an individual level. Pro-dysrhythmic risks might be genetically determined individual predisposition to TdP (Jamshidi *et al.*, 2012; Kaab *et al.*, 2012) or ambient pro-dysrhythmic factors, such as bradycardia and hypokalemia (Prandota and Iwanczak, 1983; Roden, Woosley, and Primm, 1986).

A large number of iPSC-based models of cardiac channelopathies have been established during the last few years. Moretti *et al.* (2010) generated iPSCs from patients with long-QT syndrome type 1, which is an arrhythmogenic syndrome, caused by abnormal function of the repolarizing potassium channel, due to mutations in the *KCNQ1* gene. Patient-derived iPSCs were differentiated into CMs, which faithfully reproduced the electrophysiological features of the disease, including altered channel activation and deactivation properties, and increased susceptibility to catecholamine-induced tachyarrhythmia, which could be attenuated successfully by β -blockade.

Itzhaki *et al.* (2011) derived CMs from disease-specific iPS cells, with *KCNH2 A614V* mutation, causing long-QT syndrome 2 (LQTS2). LQTS iPSC-CMs showed prolonged action potential duration because of a significant reduction in the cardiac potassium current I(Kr), which is characteristic to the disease phenotype. Both the atrial-like and ventricular-like LQTS iPSC-CMs showed marked arrhythmogenicity. Additionally, LQTS iPSC-CMs were used to investigate the potency of

existing and novel pharmacological agents to aggravate (potassium-channel blockers) or ameliorate (calcium-channel blockers, KATP-channel openers, and late sodium channel blockers) the disease phenotype, further demonstrating the potential of disease-specific iPSC-CM-based applications.

Matsa *et al.* (2011) examined CMs differentiated from a type 2 LQTS-specific iPSC obtained from a patient with a *KCNH2 G1681A* mutation. LQTS2-hiPSC-CMs showed type 2 LQTS-specific prolongation of the action potential, and responded appropriately to several clinically used drugs, demonstrating the competence of this model in disease investigation and drug testing.

Yazawa *et al.* generated hiPS cells from a patient suffering from Timothy syndrome (TS), a genetic disorder caused by a missense mutation in the gene encoding the L-type calcium channel Ca(V)1.2, which leads to cardiac arrhythmias, prolongation of the QT interval (LQTS), ventricular tachycardia, and autism (Yazawa and Dolmetsch, 2013). The TS iPSC-derived ventricular-like CMs exhibited irregular electric activity, abnormal calcium transients, and deficits in contraction because of the TS mutation. Roscovitine—a compound that increases the voltage-dependent inactivation of Ca(V)1.2—was found to be able to rescue the pathogenic phenotypes of these cells (Yazawa *et al.*, 2011).

For studying the molecular mechanisms of catecholaminergic polymorphic ventricular tachycardia (CPVT), and for the development of new therapeutic strategies, Jung *et al.* (2012b) generated hiPSCs from a CPVT1 patient. CPVT is an inherited rhythm disorder characterized by stress-induced ventricular arrhythmias in young patients with structurally normal hearts. Two genetic forms of CPVT have been described, one is caused by the autosomal dominant mutations in the cardiac ryanodine receptor, *RYR2* (CPVT1; Priori *et al.*, 2001), and the other by the recessive mutations in calsequestrin (CPVT2; Postma *et al.*, 2002).

Patient-specific iPSC-derived CMs showed increased frequency and duration of elementary Ca²⁺ sparks after catecholaminergic stress provoked by isoproterenol, as compared to control myocytes. Dantolene, a muscle relaxant, and currently the only specific treatment for malignant hyperthermia, restored normal Ca²⁺ spark properties and rescued the arrhythmogenic phenotype in patient-specific iPSC-derived CMs, providing a potential novel drug for CPVT.

Establishment of an adult-onset disease model, using a patient-specific iPS cell line, was first achieved by Kim *et al.* (2013). iPSC line was derived from a patient with arrhythmogenic right ventricular dysplasia/cardiomyopathy (ARVD/C), an inherited heart disease, causing cardiomyocyte loss, arrhythmia, and sudden cardiac death. Approximately 50–60% of patients with ARVD/C have an identifiable pathogenic gene mutation related to cardiac desmosome (Murray, 2012). Although ARVD/C iPSC-CMs demonstrated abnormal plakoglobin nuclear translocation and decreased β -catenin activity, these abnormal features were insufficient to reproduce the pathological phenotypes of ARVD/C. To fully recapitulate adult disease phenotypes, the embryonic/glycolytic state of the relatively immature ARVD/C iPSC-CMs had to be altered to achieve adult-like metabolism. Using this model, it could be demonstrated for the first time that induction of adult-like metabolism has a critical role in ARVD/C pathologies.

Carvajal-Vergara *et al.* (2010) generated iPSCs from patients with LEOPARD syndrome, a rare autosomal-dominant developmental disorder, mainly characterized by skin, facial, and cardiac anomalies. The *in-vitro*-derived CMs from LEOPARD syndrome iPSCs were larger, had a higher degree of sarcomeric organization, and a preferential localization of NFATC4 in the nucleus, as compared to wild-type iPSCs derived from a healthy donor. These features relate to a potential hypertrophic state, identical with the hypertrophic cardiomyopathy phenotype commonly observed in these patients (Sarkozy, Digilio, and Dallapiccola, 2008).

Huang *et al.* (2011) generated hiPSCs modeling glycogen storage disease type II or Pompe disease (PomD-iPSCs) and examined the applicability of the *in vitro* model for studying pathogenesis and drug effects. The human Pompe disease is a rare inherited disorder caused by autosomal recessive mutations in the acid alpha-glucosidase (GAA) gene and, consequently, the deficiency of acid maltase (Richard, Douillard-Guilloux, and Caillaud, 2011). Patients suffering from Pompe disease exhibit severe hypotonia associated with hypertrophic cardiomyopathy or progressive muscle weakness. Standard treatment of this disease is enzyme replacement therapy, although the therapeutic applications of the recombinant human GAA involve several limitations (Angelini and Semplicini, 2012).

The generated PomD-iPSCs were differentiated to cardiomyocytes-like cells (CMLCs), which showed high levels of glycogen and several ultrastructural aberrations, characteristic to the pathological phenotypes of the Pompe disease. Recombinant human GAA was able to rescue the Pompe disease phenotype, while L-carnitine treatment reduced defective cellular respiration in PomD-iPSC-derived CMLCs. The authors suggested that the above-mentioned disease model is suitable for modeling the Pompe disease and for developing novel therapies.

In some genetic disorders, cardiovascular symptoms are caused by altered properties of SMCs. Kinnear *et al.* (2013) generated a hiPSC-based *in vitro* model of the Williams–Beuren syndrome (WBS), a rare genetic multisystem disorder in which cardiovascular abnormalities are largely attributable to the deletion of the elastin-containing region on chromosome 7q11.23, causing elastin haploinsufficiency, and further leading to increased vascular SMC proliferation and stenoses (Pober, 2010). The WBS iPSC-based model could recapitulate the phenotype of the disease, as WBS iPS cell-derived SMCs possessed an immature proliferative phenotype, and the functional and contractile properties were diminished. The ability of two pharmaceutical agents, synthetic elastin-binding protein ligand 2 (EBPL2) and the antiproliferative drug rapamycin, was tested to find whether any of them rescues the disease phenotype. Both the elastin ligand and the rapamycin decreased the smooth muscle proliferation rate and enhanced differentiation; however, only rapamycin could fully rescue the pathological SMC phenotype. These results clearly suggest that this model system is suitable for testing therapeutic agents for patients with WBS and studying molecular mechanisms underlying the disease.

Ge *et al.* (2012) generated iPSC-derived SMCs from a patient with supralvalvular aortic stenosis (SVAS), a systemic elastin arteriopathy, caused by mutations in the elastin (*ELN*) gene, and further leading to abnormal proliferation of vascular SMCs (Stamm *et al.*, 2001). SVAS might be present in a nonsyndromic condition or in syndromic conditions such as WBS. The iPSC-based model of the disease recapitulated the key pathological features of patients with SVAS, as a significantly higher proliferation rate could be detected in SVAS iPSC-SMCs than in control iPSC-SMCs. It was also shown that the hyperproliferation of SVAS iPSC-SMCs was associated with elevated activity of

extracellular-signal-regulated kinase 1/2. Furthermore, the addition of elastin recombinant protein or the enhancement of small GTPase RhoA signaling rescued the formation of smooth muscle α -actin filament bundles in SVAS iPSC-SMCs, demonstrating the applicability of the SVAS hiPSC-based model system as a tool for drug discovery.

Ho *et al.* (2011) generated iPSCs from patients with distinct laminopathies bearing heterogeneous mutations in the gene encoding lamin A/C to study genotype–phenotype correlation in the respective diseases. Fibroblasts were obtained from a patient with inherited dilated cardiomyopathy and from two patients with distinct accelerated forms of aging, a typical Werner syndrome and a Hutchinson–Gilford progeria syndrome (HGPS). These are rare human genetic diseases, typically caused by mutations in codon 608 (*C1804T*) of the gene encoding lamin A and C, resulting in abnormal accumulation of progerin (Trigueros-Motos *et al.*, 2011). Besides several other symptoms, patients with HGPS exhibit cardiovascular alterations and are exposed to high risk of developing myocardial infarction or stroke. Secondary fibroblasts differentiated from the generated iPS cells and recapitulated the disease phenotype, which enabled the use of this model system for testing compounds aimed to correct lamin A/C-induced abnormalities.

As a summary, human iPSC-based *in vitro* model systems (summarized in Table 2) provide useful tools to understand the reasons for disease evolution and molecular mechanisms in the background and give an opportunity to discover and develop new potent pharmaceuticals. However, the application of hiPSC-CM-based cardiac disease models in cardiotoxicity screens is also required, as adverse drug reactions are more common in patients with preexisting heart disease than in the general population.

Liang *et al.* (2013) investigated the drug-induced cardiotoxicity profiles of hiPSC-CMs derived from healthy individuals and patients with hereditary LQTS, familial hypertrophic cardiomyopathy, or familial dilated cardiomyopathy, to model differences in susceptibility to cardiac drug toxicity for patients with different genetic backgrounds. The results of Liang *et al.* indicate that healthy and disease-specific hiPSC-CMs exhibit different susceptibilities to cardiotoxic drugs as compared to their normal counterparts and that the use of disease-specific hiPSC-CMs may predict adverse

Table 2. Summary of human cardiac-disease-specific iPSCs.

Disease	Molecular defect	Disease phenotype	Cell type	Methods	Factors	References
ARVD/C	Mutation in <i>PKP2</i> gene (plakophilin-2)	Cardiomyocyte loss, ventricular arrhythmia	Fibroblast	Retrovirus	OSKM	Kim <i>et al.</i> (2013)
CPVT1	Mutation in <i>RYR2</i> gene (cardiac ryanodine receptor)	Elevated diastolic Ca ²⁺ , reduced SR Ca ²⁺ , arrhythmia	Fibroblast	Retrovirus	OSKM	Jung <i>et al.</i> (2012b)
HGPS	Heterogeneous mutations in <i>LMNA</i> gene (lamin A/C)	Premature senescence in smooth muscle cells	Fibroblast	Retrovirus	OSKM	Ho <i>et al.</i> (2011)
LEOPARD syndrome	Mutation in <i>PTPN11</i> gene (SHP2 phosphatase)	Increased cardiomyocyte size	Dermal fibroblast	Retrovirus	OSKM	Carvajal-Vergara <i>et al.</i> (2010)
Long QT syndrome 1	Mutations in <i>KCNQ1</i> gene	Increased cardiomyocyte depolarization	Dermal fibroblast	Lentivirus	OSKM	Moretti <i>et al.</i> (2010)
Long QT syndrome 2	Mutations in <i>KCNH2</i> gene	Increased cardiomyocyte depolarization	Dermal fibroblast	Lentivirus Retrovirus	OSNL OSK	Matsa <i>et al.</i> (2011)
Pompe disease	Mutations in <i>GAA</i> gene (acid α -glucosidase)	High glycogen defective respiration	Fibroblast	Retrovirus	OSKMLN	Huang <i>et al.</i> (2011)
Timothy syndrome	Mutations in <i>CaV1.2</i> main L-type channel gene	Increased cardiomyocyte depolarization	Fibroblast	Retrovirus	OSKM	Yazawa and Dolmetsch (2013), Yazawa <i>et al.</i> (2011)

O, *OCT4*; S, *SOX2*; K, *KLF4*; M, *C-MYC*; N, *NANOG*; and L, *LIN28*.

drug responses more accurately than healthy control hPSC-CMs in the case of individuals at high risk for adverse cardiac drug reactions.

While these studies provide convincing evidence that disease-specific iPSCs are suitable *in vitro* models of several cardiac disorders, the generated cardiac lineages are relatively immature (Priori *et al.*, 2013), which can influence the evolving adult disease phenotype and drug responses as well. Accordingly, using iPSC-CMs to model an adult-onset heart disease remains a challenge. However, the most crucial challenge remains translating drug testing results into clinical applications.

8 FUTURE PERSPECTIVES

The application of CMs differentiated from human pluripotent stem cells for cardiac safety and drug discovery holds great promise. However, this promise will only meet our expectations when some

further important steps will be achieved. As a major task, pure and homogenous populations of human CMs with mature, adult-like phenotypes have to be prepared. This step should also involve the establishment of *in vitro* systems reliably recapitulating *in vivo* cardiotoxic effects or disease mechanisms in the human heart. For efficient drug screening, the most promising potential methodologies are to establish scalable cultures of human, pluripotent stem-cell-derived or directly reprogrammed CMs in large quantities. The direct conversion of CMs from human somatic cells by defined factors, without going through a pluripotent state, is a challenging possibility.

Current cardiotoxicity and drug discovery screening methods generally cannot address patient-specific properties. These include genetic variations inducing the evolvement of an individual phenotype of a certain disease or altering the reaction of healthy CMs to drugs otherwise safely applicable in the majority of patients. The recently

established methods, such as the generation of human patient- and disease-specific CMs from pluripotent stem cells, or directly reprogramming of CMs from mature somatic cells, should provide a major step toward personalized drug development. Adverse effects evoked by noncompatible drugs or the lack of drug efficacy in certain patients could be avoided, and the dosage of compatible drugs could be optimized to the individuals.

The generation of patient-derived CMs also offers the possibility to establish *in vitro* models of cardiac diseases, faithfully recapitulating specific disease phenotypes. Cardiac disease model systems can be obtained from genetically modified CMs, derived from pluripotent stem cells, reflecting specific inheritable disease conditions. Moreover, early stages of the disease-specific cardiogenesis can be studied, revealing the underlying mechanisms and enabling the discovery of new drugs potentially rescuing the disease phenotype even before birth. This chapter summarizes the efforts and promising results addressing these important issues.

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Pluripotent Stem Cells as Tools to Assess Developmental Toxicity: Diversity Instead of Consolidation

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1 INTRODUCTION

The embryonic development of an organism represents a relatively short, yet vulnerable, span in its entire life cycle. Therefore, any teratogenic compound acting upon the developing organism during pregnancy may severely perturb development. In the early 1960s, the thalidomide disaster demonstrated the urgent need to identify and predict the developmental toxicity of putative toxicants before being released into the environment or for human use for the first time.

Nowadays, human populations are experiencing a rapid increase in exposure to potential environmental toxicants in the form of pharmaceutical drugs, commercial chemicals, industrial by-products, and wastes that are untested for safety. Most of these chemicals need to be assessed for their health risks to determine acceptable exposure levels. *In vivo* studies according to the Organization for Economic Co-operation and Development (OECD) guidelines are currently performed to provide such data. It is estimated that 100,000 existing chemicals circulate the market in the United States and that 2000 new substances enter it each year. Apart from the tremendous associated costs, *in vivo* testing of such a magnitude of chemicals would require

enormous amounts of animals and a great deal of time. Clearly, *in vivo* testing methods would not be feasible to adequately test all current and putative substances. Hence, it is crucial to develop alternative *in vitro* methods to assess toxicity, especially teratogenicity, to test current and putative chemicals for their impact on prenatal development while at the same time reduce the use of animals and cut expenses.

Over the past 30 years, a number of *in vitro* approaches have been developed to screen chemicals for their teratogenic effects and circumvent traditional *in vivo* methods. In 2002, three of these *in vitro* embryotoxicity assays were validated according to standards of the European Centre for the Validation of Alternative Methods (ECVAM): the micromass assay (Umansky, 1966), the whole embryo culture assay (Cuthbertson and Beck, 1990), and the embryonic stem cell test (EST) (Genschow *et al.*, 2000, 2002). However, micromass and whole embryo culture assay are labor intensive and still require the killing of pregnant animals and/or embryos. The only *in vitro* mammalian-based developmental toxicity test that does not sacrifice pregnant animals is the EST, as it uses permanent cell lines, embryonic stem cells (ESCs), and 3T3 fibroblasts.

2 EMBRYONIC STEM CELLS

In recent years, scientific understanding of stem cells has greatly expanded the field of toxicology. ESCs are derived from the inner cell mass of the blastocyst-stage embryo (Figure 1). ESCs can maintain pluripotency for prolonged periods *in vitro*, have the ability to undergo sustained proliferation, and are able to differentiate and give rise to all three germ layers and thus all cells of the body (Valier, Reynolds, and Pedersen, 2004). ESCs express Oct-3/4, nanog, and Sox-2 (Chen and Daley, 2008) to maintain pluripotency, and the downregulation of these genes is associated with differentiation initiation (Rao and Orkin, 2006). The prolonged lifespan of ESCs is attributed to high telomerase activity, which is typically low in somatic cells (Thomson *et al.*, 1998). Thus, ESCs have an unlimited proliferation capacity *in vitro*, which does not hold true for adult stem cells (Hiyama and Hiyama, 2007).

For ESCs to differentiate toward a specific lineage, expression of pluripotency markers must be suppressed. In addition, chemical cues can be added to further push cells toward a particular lineage. ESCs progressing toward a particular lineage first produce a daughter cell and a progenitor cell, in an asymmetrical division (Sommer and Rao, 2002). Progenitor cells undergo a limited number of divisions (a distinction from ESCs) before terminally differentiating into a mature cell (Seaberg and van der Kooy, 2003). In a nonadherent environment, ESCs aggregate to form embryoid bodies (EBs), which comprise progenitor cells of various lineages. Owing to this ability to propagate themselves indefinitely and to mimic embryogenesis by producing a multitude of cell types, ESCs are a developmentally powerful tool for toxicological assays. For instance, cultures of spontaneously differentiated ESCs will contain contracting cardiomyocytes (Christoforou *et al.*, 2008), a feature that is exploited in the classic EST.

3 EMBRYONIC STEM CELL TEST

The EST is the most promising mammalian *in vitro* assay to predict the embryotoxic potential of test chemicals. Many congenital abnormalities occur because of the adverse affects of deleterious agents on development processes, and as the EST

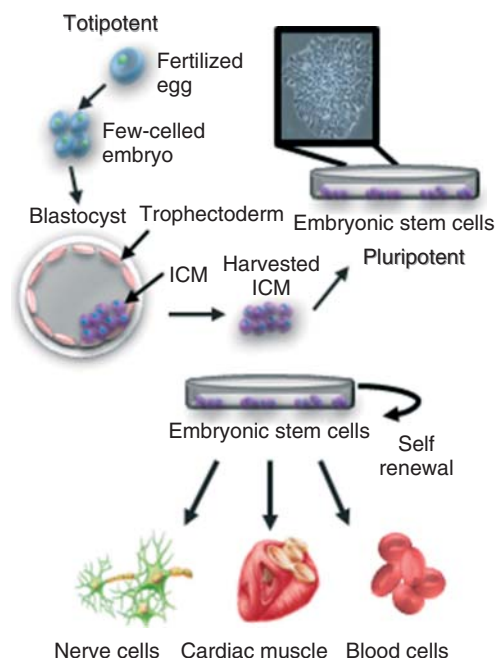


Figure 1. Embryonic stem cell derivation and differentiation. Totipotent cells (zygote) can form all types of cells including the organism and the trophoblast. Pluripotent embryonic stem cells are derived from the inner cell mass of the blastocyst-stage embryo and can form all three germ layers, but not the extra embryonic structures.

predicts teratogenic potential during this growth period, it is the ideal test. It classifies substances into three classes of *in vivo* embryotoxicity (strong, weak, and not embryotoxic) (Scholz *et al.*, 1999a; Genschow *et al.*, 2002). Murine embryonic stem cells (mESCs) are utilized in the EST to assess the embryotoxic potential of the test substance as their *in vitro* differentiation recapitulates developmental processes and is characterized by gene expression patterns indicative of *in vivo* cardiogenesis.

3T3 fibroblasts are also evaluated as they model the effect of the chemical on mature somatic cells (Figure 2). For the classification of the embryotoxic potential of the test compound into the above-named categories, three endpoints are determined: (i) cell differentiation: the concentration of the test compound that causes a 50% inhibition of the differentiation of ESCs into cardiomyocytes (ID_{50}); (ii) viability of immature cells: the concentration of the test compound resulting in a 50% decrease in the viability of ESCs [assessed by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenoltetrazolium

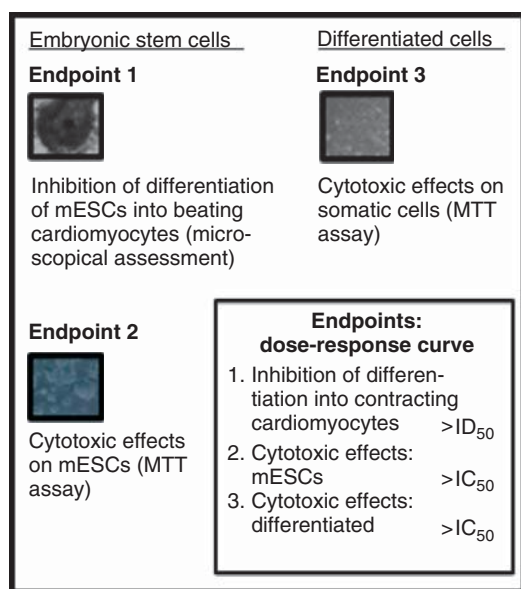


Figure 2. Embryonic stem cell test (EST). Overview of the approach and endpoints to assess the embryotoxic potential of a test chemical using mESCs and mouse fibroblasts.

bromide) assay] (IC₅₀); and (iii) viability of mature somatic cells: the concentration of the test compound resulting in a 50% decrease in the viability of 3T3 fibroblasts (IC₅₀) (Scholz *et al.*, 1999b). As previously mentioned, spontaneously differentiating EBs contain cell types of all three germ layers, amongst them beating cardiomyocytes. Indeed, the heart is the first organ to form in the developing embryo and contracting clusters of cardiomyocytes can be microscopically identified *in vitro*, which suggested that cardiomyocytes are a suitable endpoint for the EST.

In addition, the EST is the only *in vitro* test with a biostatistical prediction model that circumvents the use of animals to evaluate the embryotoxic potential of test compounds (Scholz *et al.*, 1999a,b; Genschow *et al.*, 2000, 2002, 2004). Using this prediction model, the National Centre for Documentation and Evaluation of Alternative Methods to Animal Experiments (ZEBET) at the German Federal Institute for Risk Assessment coordinated ECVAM's prevalidation and validation studies of 6 and 14 compounds, previously characterized by *in vivo* data, respectively. The prevalidation study evaluated the prediction model designed for the EST, and based on those results, the prediction model was

reevaluated with the remaining 14 test substances in the validation study (Spielmann *et al.*, 2006). The validation study resulted in a 78% correct classification of the 20 test compounds (Genschow *et al.*, 2000, 2002, 2004). In addition, the test displayed 100% predictivity for strong embryotoxic chemicals (Spielmann *et al.*, 2006). The validation study comprises an intra- and interlaboratory exploration using six strong embryotoxic substances, seven weak substances, and seven nonembryotoxic substances under blind conditions in four laboratories (Spielmann *et al.*, 2006). All four laboratories showed 100% predictivity and 85% precision of strong embryotoxic chemicals. Moreover, weak and nonembryotoxic correct classification of chemicals exhibited 83 and 75%, respectively. Furthermore, there was a correlation between *in vivo* and *in vitro* classification in the four laboratories of 85, 83, 81, and 68%. These numbers show that the test could not decipher between weak and nonembryotoxic compounds, despite being successful in identifying strong embryotoxic chemicals. Moreover, the test could not detect compounds whose metabolites act as embryotoxicants; however, the overall positive results gave the EST the green light to be considered for regulatory uses (Genschow *et al.*, 2002).

With the validation from ECVAM, some pharmaceutical and chemical companies employed the EST and found it useful for early stages of product development. When originally invented, the EST was designed for access to all laboratories, including pharmaceutical and chemical companies, with a standard, validated set of procedures. A decade later, improvements were clearly needed for the EST. Pfizer utilized ECVAM's validated EST with compounds used to validate the EST and in-house compounds (Paquette *et al.*, 2008). Their study showed that the test was robust and able to be used in other laboratories, however needed improvements. In pursuit of developing a standardized EST for all laboratories, ECVAM held a workshop in 2003 to address the future of the test. Key toxicologists determined that testing 20 compounds was not sufficient and the choice of chemicals needed to be expanded for validating the test (Spielmann *et al.*, 2006). Additionally, molecular endpoints and additional differentiation pathways, such as neural and osteogenic, needed to be included. Furthermore, it was suggested that the successful use of human embryonic stem cells (hESCs) in embryotoxicity screens would abrogate interspecies differences and

was more reliable to predict human effects, while still reducing the number of laboratory animals used. Thus, instead of further validation studies for standard use, more *in vitro* models for prediction were developed.

These modifications to the EST and new *in vitro* models were done in-house, which created diversity amongst screening methods instead of further consolidating the method. Developmental toxicology must assess the efficacy of the models to produce a high throughput coherent set of procedures for all to adapt to. However, owing to these in-house developments, the new methods, some of which are reviewed later, were not tested for inter- and intralaboratory capability and not validated although promising.

3.1 Improvements to the Embryonic Stem Cell Test

Although it was established early on that ESCs are a powerful and valuable tool to examine the embryotoxic potential of a chemical, the originally tested cardiogenic endpoint is problematic. The readout of the classic EST is based on the presence or absence of beating cardiomyocytes and requires skilled personnel to microscopically detect such cells. In addition to being a technically challenging endpoint, cardiogenesis may also not be a valid endpoint for all teratogens. For example, a variety of developmental toxicants result in limb, skeletal, or CNS (central nervous system) malformations (zur Nieden *et al.*, 2004). It was therefore deemed to be beneficial to differentiate ESCs into other lineages that can be easily tested with the EST to identify developmental toxicity in other endpoint organs (zur Nieden *et al.*, 2004). For instance, the EST is one of few tests available to study the effects of agents on skeletal development *in vitro* (zur Nieden *et al.*, 2010a, 2010b; zur Nieden and Baumgartner, 2010). Our group has established a method that abrogates the need for expensive molecular assays and utilizes morphometric image analysis to assess developmental osteotoxicity (zur Nieden *et al.*, 2010a). We have used the mouse-based assay to determine developmental osteotoxicity in pharmaceutical compounds and have also investigated developmental osteotoxicity of chloride derivatives (zur Nieden and Baumgartner, 2010).

In addition to the osteogenic endpoint, neural, chondrogenic, and endothelial endpoints were also developed to assess the embryotoxic potential on such cells (zur Nieden *et al.*, 2004; Festag *et al.*, 2007a). This ability to test additional differentiation pathways is due to advances in stem cell biology that allow for the directed differentiation of the cells into specific lineages with high yield (zur Nieden *et al.*, 2003, 2005; Buttery *et al.*, 2001; Festag *et al.*, 2007b). Interestingly, these studies highlight concentration- and tissue-specific effects of compounds. For example, at a specific concentration, retinoic acid, a classified strong teratogen, caused a 400% increase in osteoblast marker gene expression, a 50% decrease in a chondrocyte specific mRNA, and no disruption of neural or cardiomyocyte gene expression. These intriguing results underscore the importance of testing multiple differentiation pathways.

In addition to developing screens for multiple diverse differentiation pathways, laboratories made improvements to the EST by establishing molecular endpoint analyses that could enhance the predictivity of the test. Bremer *et al.* (1999) were the first to use transgenic mESCs, in which green fluorescent protein (GFP) linked to a cardiac specific promoter was used as a toxicological endpoint by quantifying the number of GFP positive cells in cultures treated with chemicals known to affect *in vivo* development with flow cytometry. At the same time, flow cytometric methods provide the ability of high-throughput screening and are a more robust and reproducible screening system. Seiler *et al.* (2002) also developed a quicker and more reproducible screen by discovering that the flow-cytometry-based detection of tissue-specific markers in compound-treated mESCs could accurately predict teratogenicity. In addition to protein expression, we reported that changes in marker gene expression in treated cells as quantified by RT-PCR (reverse transcription polymerase chain reaction) are predictive of the teratogenic potential of the compound. Therefore, the analysis of gene expression is a promising tool to study developmental toxicology because embryogenesis is aided by the changes in the regulation of genes. An advantage of determining which genes are affected by specific compounds is that it can lead to further elucidation of the underlying mechanisms of the teratogenic effect. Aside from tissue-specific protein and gene expression, the spontaneous extracellular field

potential of differentiating cardiomyocytes measured with a microelectrode array system was found to be inhibited by embryotoxic chemicals (Koseki *et al.*, 2010). Such promising studies underline the fact that it is worthwhile to include additional endpoints that enhance the predictivity of the EST.

Other improvements to the EST were attempts to reduce test duration. Although still quicker than an *in vivo* experiment, the first beating cardiomyocytes emerge at 9–10 days after *in vitro* culture, which does not facilitate the high throughput demands of testing the increased number of toxicants in the environment. It is necessary to develop an *in vitro* screening assay that reduces the time it takes to have the results in hand. Cardiac protein and gene expression endpoints mentioned above not only served the purpose of increasing predictivity but also allowed earlier detection of teratogenic effects on cardiogenesis. As such, quantitative PCR analysis of cardiac-specific myosin heavy chain (MHC) expression reduced the test to 8 days and still accurately assessed chemicals in accordance with their *in vivo* potential (zur Nieden *et al.*, 2001). In addition, the FACS-EST (fluorescence-activated cell sorting embryonic stem cell test) produced the same sensitivity as the validated EST, but reduced the length of time of the assay to 7 days (Buesen *et al.*, 2009; Seiler *et al.*, 2004). While one would expect the appearance of mRNAs before the appearance of protein, the discrepancy in timing between these two studies may potentially be explained by the different serum lots used for the differentiation of the ESCs.

Similar attempts were made by our group to shorten the duration of skeletal endpoint differentiations (zur Nieden *et al.*, 2010b). While we were successful in assessing osteotoxicity at the level of mRNA expression of bone marker genes, these endpoints could not be analyzed until 30 days of osteogenic differentiation. To shorten the assay, we have made significant improvements to the culturing method and can now assess the teratogenic potential of a chemical in 14 days (zur Nieden *et al.*, 2010b). We currently use a variation of this method for human pluripotent stem cell induction to osteoblasts and assessment of toxicants (Figure 3).

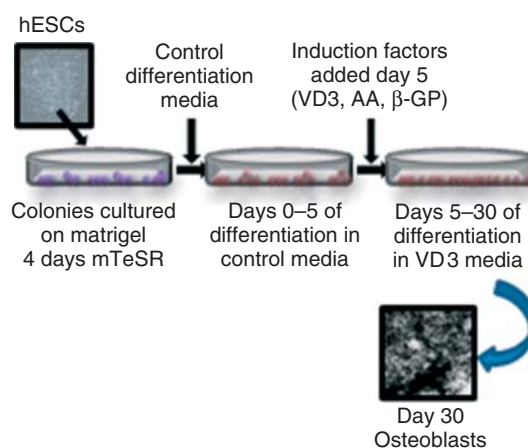


Figure 3. Human ESC osteogenic induction. Osteogenic differentiation is initiated after the cultures become confluent (designated d0) with Dulbecco's Modified Eagle Medium (DMEM) and fetal bovine serum (FBS). On day 5, 1,25α(OH)₂ vitamin D₃ (VD₃), β-glycerolphosphate (GP), and ascorbic acid (AA) are added to the medium to direct cells toward the osteogenic lineage. The presence of calcification is measurable on day 14, and by day 30, the culture is predominantly osteoblasts.

3.2 Beyond the Embryonic Stem Cell Test

With lack of consensus on which *in vitro* tests or endpoints are suitable for determining the embryotoxic potential of a compound, more developmental toxicity screens are being developed. For instance, the adherent cell differentiation and cytotoxicity (ACDC) assay is a murine-ESC-based model designed to (i) evaluate the effects of chemical exposure on stem cell number and differentiation using a single culture technique, (ii) quantify markers of cell number and cardiomyocyte differentiation, and (iii) provide an assessment of multiple differentiation markers (Barrier *et al.*, 2011). The model abrogates the use of the EB-based differentiation system for an adherent method, which simplifies the culturing technique and is completed in 9 days. An advantage of this assay is the way cell number and cardiomyocyte differentiation are assessed; both are done after fixation on the same cells in the same culture wells. Cell number and MHC protein were both measured using the in-cell western protocol developed to use with the Li-Cor Odyssey infrared imaging system. This system measures two endpoints in each well with two infrared channels at 700 and 800 nm. In this study, Barrier *et al.* (2011) used the 700-nm channel to quantify the relative cell

number and the 800-nm channel to detect the MHC signal. This assay is beneficial in that it simplifies culture techniques and can assess two endpoints in one vessel—creating a high throughput assay that has the potential of full automation (Barrier *et al.*, 2011). Although promising, this assay does not include a prediction model such as the EST.

More than 90% of chemicals that enter the body are metabolized in the liver into other forms, metabolites, which are a potential culprit that skews development (Wilkinson, 2005; Kleinstruer *et al.*, 2011). Primary rat hepatocytes have been utilized to metabolize the compound *in vitro* before analysis. However, these cells are difficult to maintain and are plagued by interspecies variation (Greenhough, Medine, and Hay, 2010). Furthermore, cells are typically harvested from rats, which would not reduce animal use. Other currently available systems for mimicking liver metabolism include the use of S9 liver extracts, transgenic cell lines, hepatocyte-like cell monolayers, and 3D organotypic cultures (Adler *et al.*, 2011; Esch, King, and Shuler, 2011; Giri *et al.*, 2011; Landsiedel *et al.*, 2011). Pluripotent stem cell research has enabled the ability to derive hepatocyte-like cells from hESCs; however, the yield and efficiency are low and gene expression profiles vary (Greenhough, Medine, and Hay, 2010). With a clear need for metabolism models, North America and the European Union have created virtual liver initiatives. The US EPA (Environmental Protection Agency) Virtual Liver Project (Environmental Protection Agency (EPA), 2009) uses mathematical models to predict liver toxicity based on molecular targets, signaling pathways, and toxicity at the organ and organismal levels.

Similar to interspecies differences in the ability to metabolize substances, individuals too differ in their ability to metabolize chemicals (Xie *et al.*, 2001), making it difficult to reliably predict toxicity using cells derived from a single donor (Greenhough, Medine, and Hay, 2010). Here, the development of induced pluripotent stem cells (iPSCs) has revolutionized stem-cell-based toxicity screening. iPSCs are generated by the reprogramming of somatic cells through the introduction of four key transcription factors: Sox-2, Klf-4, Oct4, and c-Myc (Takahashi and Yamanaka, 2006; Takahashi *et al.*, 2007). These cells possess a number of advantages over hESCs, including patient-specific cell-based drug testing and are free from the ethical concerns associated with the use of blastocysts. The creation

of liver toxicity screening technologies utilizing iPSC-derived hepatocyte-like cells would allow investigation into the effects of single nucleotide polymorphisms on drug metabolism and toxicity (Greenhough, Medine, and Hay, 2010).

4 MODEL ORGANISM

Murine ESCs are able to recapitulate hallmarks of embryogenesis and murine-ESC-based embryotoxicity assays are thus a great tool to elucidate mechanisms of developmental toxicity. However, mouse models are not able to entirely mimic human development. Moreover, culturing methods and expression of certain genes differ between murine and human ESCs. For instance, human ESCs form flat colonies, do not survive as single cells, and require basic fibroblast growth factor (bFGF) in the culture medium to maintain an undifferentiated state, whereas murine ESCs have three-dimensional colonies, survive as single cells, and leukemia inhibitory factor is added to culture medium. These differences limit the transferability of predictions for human use made based on mouse cells. Indeed, *in vivo* developmental toxicity studies have shown high interspecies differences, despite the usefulness of the murine-based EST (Hurt, Cappon, and Browning, 2003). Specifically, thalidomide has shown no teratogenic effects in mice, but causes profound human congenital malformations (Gilbert, 2003; Fratta, Sigg, and Maiorana, 1965; Brent and Holmes, 1988). Therefore, integrating human stem cells into developmental toxicity assays is necessary to accurately account for the human response to putative toxicants. In addition, these cells are highly attractive to the pharmaceutical industry in facilitating drug discovery development.

As a first step, Buzanska *et al.* (2009) utilized a human neural stem cell line derived from umbilical cord blood (HUCB-NSC) as a model to test developmental neurotoxicity. The study showed that differently staged HUCB-NSCs were able to discriminate between neurotoxic and non-neurotoxic chemicals. The endpoints (cell viability, proliferation, apoptosis, and expression of cell-specific markers) were assessed 48 h after exposure to test chemicals of early and late stages of HUCB-NSC. Cell viability was measured by the live/dead viability/cytotoxicity (LDVC) assay, MTT assay, or the resazurin reduction assay. Nuclear chromatin

staining and immunocytochemical labeling for stage-specific markers were used to determine cell proliferation, apoptosis, and neural differentiation. The LDVC assay allowed for the rapid discrimination of live and dead cells microscopically or with flow cytometric methods. The MTT and resazurin reduction assays are both colorimetric assays that rely on the ability of mitochondria to reduce MTT or resazurin, respectively, to measure viable cells. The downside of such assays is that they are not true viability tests because they measure mitochondrial potential, which could also be altered due to changes in cellular metabolism. Furthermore, apoptotic cell identification could benefit from additional endpoints, such as caspase-3 quantification, to support chromatin condensation data.

Although some of the endpoint assays in the HUCB-NSC study could clearly be improved, an advantage of their approach was the usage of human cells. Advantageous from the standpoint of choice of test species, their approach differentiates neural progenitors as opposed to less committed cells and therefore evaluates particular stages of development only. In 2011, Colleoni *et al.* (2011) developed an alternative approach to assess neural teratogenicity using pluripotent human ESCs. The group differentiated human ESCs into neural rosettes, which represent neural plate and neural tube development, and exposed the cells to retinoic acid. This treatment would induce neural tube defects, which is one of the most common birth defects, affecting nearly 0.5–2 newborns per 1000 (Colleoni *et al.*, 2011). Their microarray analysis and qPCR data suggested dose-dependent misregulation of genes involved in neural tube formation and closure, such as the homeobox family genes and Otx2. Furthermore, this *in vitro* modulation of neurulation markers in response to retinoic acid correlated with gene expression responses associated with *in vivo* retinoic acid exposure. Despite being a first promising step toward a predictive human-based *in vitro* model, this approach requires further validation of other teratogenic chemicals and lacks a prediction model to classify substances such as the one used in the classic EST.

In a similar approach, West *et al.* (2010) developed a hESC-based model that used metabolomics to detect small molecule changes in response to teratogen exposure, a concept that was first introduced by Cezar *et al.* (2007). In drug treated and untreated cells, the group reported significant differences in

metabolite abundances, with asymmetric dimethylarginine (ADMA) being the most predictive of teratogenicity, particularly neural tube closure. The discovered alteration of ADMA upon teratogen exposure suggests that it is a potential biomarker for neural tube defects when modified. However, further exploration is needed to detect biomarkers in toxicity of differentiation pathways. Novel approaches such as this will potentially elucidate the underlying mechanisms of developmental toxicity.

5 CONCLUSION

There are many demands in the field of developmental and predictive toxicology to create an *in vitro* system that meets the standards of academic and industrial toxicity screening. Although *in vitro* screening methods in developmental toxicology have tremendously advanced, there is still a lack of agreement on what constitutes a developmental toxicant and how these characteristics such as physiological endpoints, maternal toxicity, dose magnitude, and duration can be translated into, or derived from the *in vitro* model (Castoldi *et al.*, 2008). The EST is the most promising validated *in vitro* developmental toxicity model, though its endpoints as well as the use of murine cells limit the utility of the test. These downfalls have created a frenzy of improvements and have led to new *in vitro* approaches (some including hESC models). New *in vitro* efforts would benefit the EST if toxicologists focused on standardizing a set of assays to assess developmental toxicity instead of creating diversity amongst the field.

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Impact of Various Nanosystems on Stem Cell Physiology

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1 INTRODUCTION

In the past few decades, significant progress has been made in the study of the interactions of nanomaterials and biological systems. Owing to their unique chemical, electronic, and optical properties, nanoparticles have attracted special attention. By simply changing their surface chemistry, they can be adapted for applications in a wide variety of fields, including catalysis (Crooks *et al.*, 2001), electronics (Ko *et al.*, 2007), biosensing (Luo *et al.*, 2006), and, most importantly, medicine (Elmes *et al.*, 2011; Galanzha *et al.*, 2013; Iancu *et al.*, 2011; Mocan *et al.*, 2011; Orza *et al.*, 2010). The benefits of using nanotechnology for medical applications provide hope for improving the management of traditional medicine. There is a broad spectrum of reports concerning the use of nanotechnology for both the diagnosis and treatment of different types of cancer, as well as for the treatment of Parkinson's (Modi, Pillay, and Choonara, 2010) and cardiovascular disease (Buxton, 2009) and in tissue engineering (Orza *et al.*, 2011). Nanotechnology makes it possible to achieve the targeted delivery of drugs (Farokhzad and Langer, 2009), genes (Ganta *et al.*, 2008), or other biologically active molecules (Liu *et al.*, 2008a) and to create highly sensitive biosensors (Luo *et al.*, 2006), high-speed cell sorters (Radisic, Iyer, and Murthy, 2006), and other photoacoustic or photothermal therapeutics (Yang *et al.*, 2012; Wang *et al.*, 2004; Kim *et al.*, 2010).

There are several reasons why nanomaterials have such a broad range of applications: (i) they are endowed with distinctive features that differ from those of bulk materials, such as size, surface chemistry, and, in some cases, biological inertness (Kiely and Houston, 1998); (ii) they are smaller than subcellular components by an order of magnitude; and (iii) their high surface-to-volume ratio gives them the ability to be multifunctionalized, which, in turn, enables them to be used in multiple therapeutic approaches (Aillon *et al.*, 2009).

Some examples of nanomaterials that have been synthesized for biomedical purposes are semiconductor quantum dots (QDs), iron oxide nanoparticles (IONPs), noble nanoparticles, carbon nanotubes, graphenes, and so on. On the basis of their fluorescent properties, bright and stable light emission, and tunable size, semiconductor QDs have been successfully employed for optical imaging *in vivo* and *in vitro* (Michalet *et al.*, 2005; Zhang *et al.*, 2013b). These properties enable QDs to be used as substitutes for traditional fluorescent molecules—which are unstable when exposed to light and bleach after only a few minutes. QDs have been used in cancer imaging and have shown high fluorescence, as reported by Harisinghani *et al.* (2003), who imaged *in vivo* tumors in mice by injecting antibody-conjugated QDs. Moreover, IONPs can be used both for delivery detection and the magnetic targeting of an area of interest. They have also been used to identify some small-sized lymph node

metastases that would otherwise have been undetectable (Laurent *et al.*, 2008). Carbon nanotubes and graphenes are other classes of nanomaterials that can be used for both cancer therapy and regenerative medicine (Pryzhkova, 2013; Ali-Boucetta *et al.*, 2013; Singh *et al.*, 2012). Another class of nanoparticles that has been used in human health since the Middle Ages are colloidal gold nanoparticles (GNPs). These nanoparticles have been shown to have excellent biological inertness. When cells are cultured in their presence, the proliferation rate, protein synthesis, and morphological structures are insensitive to concentration and exposure time (Lu *et al.*, 2010). In addition, on the basis of their strong thiol bond, their surface can be decorated covalently or noncovalently with a variety of biomolecules (DeLong *et al.*, 2010) specific to the requirements of the application.

Recent studies have focused on the interaction of nanoparticles and stem cells (Xu *et al.*, 2013; Smith *et al.*, 2012; Li *et al.*, 2013; Tsai *et al.*, 2013; Wang *et al.*, 2013a; Mironava *et al.*, 2014; Soenen *et al.*, 2012). Stem cells possess self-renewal properties and the capacity to differentiate into other lineages. Numerous types of stem cells have been isolated from different parts of the organism, both from mice and humans (Morrison and Weissman, 1994a).

Cancer stem cells (CSCs) are similar to other stem cells: they have self-renewal properties, and they are pluripotent. CSCs are most likely formed by mutations in normal stem cells or progenitor cells (Clarke and Fuller, 2006; Reya *et al.*, 2001; Fearon and Vogelstein, 1990; Clarke, 2005). CSCs have been found to be resistant to chemotherapeutics and radiotherapeutics (Till and McCulloch, 1961). Therefore, advanced treatments that could target these cells must be developed. The markers that have been identified on the surface of CSCs are as follows: CD24, CD44, CD133, A2B5, CD166, EpCAM, and integrins (Kondo *et al.*, 2003; Spangrude, Heimfeld, and Weissman, 1988; Morrison and Weissman, 1994b; Morrison *et al.*, 1997; Kiel *et al.*, 2005). Smith *et al.* (2008) have recently targeted hepatocellular and gastric cancers using an antibody-drug conjugate—murine antihuman CD133 antibody conjugated with monomethyl auristatin F.

On the basis of their complexity, it is crucial to understand the biology of CSCs. It is also important to evaluate the toxicity attributed to the

exposure of these cells to various nanomaterials. Nanotechnology offers a multitude of advantages, but its potential impact on human health must be thoroughly assessed. Understanding the toxicity of various nanomaterials will provide information about risks to be avoided, such as inflammatory, genotoxic, oxidative, or cytotoxic effects. The internalization of nanomaterials by living systems raises big concerns; based on their small size, they have the ability to penetrate biological membranes, therefore affecting cell physiology (Alkilany and Murphy, 2010; Babin *et al.*, 2013; Galeone *et al.*, 2012). For stem cells, this consideration is of extreme importance because the mechanism through which the nanoparticles affect their self-renewal and differentiation is unknown. Moreover, limited data concerning the toxicity of nanomaterials on adult stem cells is available. The side effects of nanoparticles' toxicity include cell maturation and the inhibition of proliferation that can lead to different diseases, such as cancer. For example, Braydich-Stolle *et al.* (2005) described the effect of chemicals on mammalian germline stem cells and their heritable structural and chromosomal aberration.

In this work, we address the question of the safety of nanoparticles for use in medicine. We focus on the effects of GNPs, IONPs, single-walled carbon nanotubes (SWCNTs), and multiwalled carbon nanotubes (MWCNTs) on stem cell cultures. Light microscopy, cell proliferation, and standard cytotoxicity assays are used for this purpose. In addition, based on our previous research and on the available literature, we identify several parameters (e.g., size, surface area, and concentration) that can be controlled in order to optimize and limit the nanoparticles' cellular toxicity.

2 CHALLENGES CONCERNING THE TOXICITY OF NANOMATERIALS

It is very important to synthesize nanostructures with a variety of architectures and to assess their toxicity in order to use them for diverse bioapplications. Moreover, it is necessary to understand that, in addition to their size and shape, their toxicity also depends on their composition and stability.

Various types of nanoparticles of different metals have been synthesized (Tuttolomondo *et al.*, 2013; Boucher *et al.*, 2013; Qian *et al.*, 2012; Ding *et al.*,

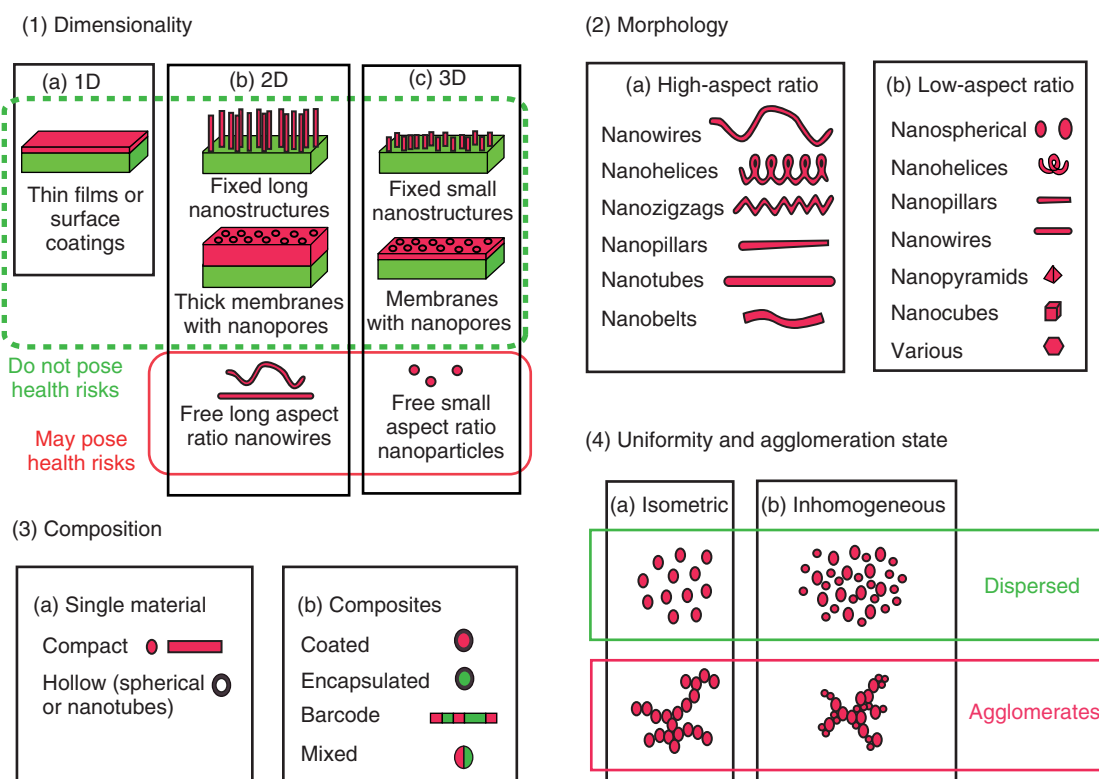


Figure 1. Classification of nanostructured materials based on their dimensions, morphology, composition, uniformity, and agglomeration. [Buzea, C, Pacheco, I, Robbie, K. (2007). Reproduced with permission from Springer.]

2012; Hafez and El-Fadaly, 2012; Xue *et al.*, 2012; Laurent *et al.*, 2008; Zhang *et al.*, 2013a; Luo *et al.*, 2013). They are generally classified based on their size, morphology, monodispersity, composition and colloidal stability, and surface chemistry (Richards and Ivanisevic, 2012). These are the main parameters that determine the final properties of the nanoparticles and tightly control their biocompatibility. Figure 1 shows the classification of nanostructured materials based on their size, shape, composition, monodispersity, and stability (Buzea, Pacheco, and Robbie, 2007).

Many types of nanoparticles have been demonstrated to be nontoxic (Luo *et al.*, 2013; Buzea, Pacheco, and Robbie, 2007; Goodman *et al.*, 2004; Derfus, Chan, and Bhatia, 2004) to be beneficial in health care (Bosi *et al.*, 2003; Schubert *et al.*, 2006). However, other nanomaterials have been reported to be toxic (Fenoglio *et al.*, 2013, 2006; Sayes *et al.*, 2007). There are many methods that can decrease their toxicity, such as decorating their surface with a

thin shell of a biocompatible material. Nevertheless, this raises the possibility that, if the properties of the nanoparticles have changed as a result of functionalization, a new composite material may have been obtained.

On the basis of the complexity of the subject, complete toxicological studies on various classes of nanomaterials having different dimensions, morphologies, compositions, and states (uniformity vs agglomeration) must be conducted in order to achieve accurate information about the safety of their use in biomedical applications.

One of the most important questions when studying the toxicity of nanomaterials toxicity concerns their unique physical properties. Do nanomaterials produce a uniquely aggressive range of toxicity compared with bulk materials? Are they more bio-reactive and more potent? In order to answer these questions, there are several factors that must be considered when testing their toxicity: the type of the nanomaterial, cell-type surface chemistry

(functional groups and different coatings), physicochemical parameters (diameter, surface charge and area, and topography), the concentration of nanoparticles, the size of nanoparticles, their surface chemistry, their degree of purification, and the exposure time and the types of assays used.

One limitation of current studies is that the majority are focused on the cytotoxic effect of only one type of nanoparticle as analyzed using only one assay. Thus, the comparison between different studies is limited, and the actual safety of the application of those nanomaterials in the biomedical field remains unclear. In the past few years, an increased number of reports (Fenoglio *et al.*, 2006; Sayes *et al.*, 2007; Xu *et al.*, 2013; Smith *et al.*, 2012) have discussed the cytotoxicity of nanomaterials in cell cultures, but only a few of have focused of the impact of nanomaterials on stem cells.

3 PHYSICOCHEMICAL PARAMETERS OF THE NANOPARTICLES VERSUS TOXICITY

3.1 Composition and Surface Chemistry

When incubating nanoparticles with living cells, they are exposed to a variety of components from the cell culture media that lead to nano-specific interactions between the nanoparticles' surface and serum proteins. These interactions have a major effect on the nanoparticles' uptake and toxicity. In the case of IONPs, an increase in uptake has been observed after 12 h of incubation (Chen *et al.*, 2009). In addition, significant uptake differences have been observed when the nanoparticles' surface was coated with ligands containing the COOH, NH₂, and polyethylene glycol (PEG) groups. Interestingly, the uptake differences disappear if the exposure of the nanoparticles to cell media lasts for more than several hours—probably because of the increasing absorption of serum proteins on their surface (Chen *et al.*, 2009). Ehrenberg *et al.* (2009) have found that uptake is governed by the capacity of the nanoparticles to absorb serum proteins. Giljohann *et al.* (2007) observed an increase in the size of 13 nm GNPs functionalized with oligonucleotides, after their exposure, and they concluded that the amount of nucleotide from the surface of the nanoparticles played an important role in the nanoparticle–cell interactions (Giljohann *et al.*, 2007). However, PEG seems to be the only

ligand that prevents the nonspecific interaction (Free, Shaw, and Levy, 2009). It has been shown that, in normal cell lines, the PEG ligand greatly reduces nanoparticle uptake. The uptake of GNPs functionalized with PEG 5000 was completely blocked; however, if the surface was partially decorated with signaling peptides, uptake would still occur (Liu *et al.*, 2008b). However, contradictory data can be found in the literature. For example, Gu *et al.* have reported a low uptake of 3–4 nm GNPs functionalized with PEG after 30 h (Chithrani, Ghazani, and Chan, 2006). They also presented evidence that the nanoparticles accumulated in the nucleus. Moreover, Liu *et al.* (2008b) have demonstrated an increase in the uptake of 4.7-nm pegylated GNPs in CT26 cells. One explanation for this conflicting data could be that the nanoparticles were not efficiently functionalized. It is believed that the amino-propyl group could play an important role in the cell–PEG nanoparticle interaction, but further investigation will be necessary in order to investigate the blocking of uptake by the PEG molecule.

Wet synthesis involves the use of various ligands to control the shape and the stability of the final nanoparticle. As discussed previously, the surface ligand influences the interaction between the cells and nanoparticles through their physicochemical properties, thus playing a primary role in their response upon exposure. Thus, for safe use in future applications, it is of great importance to evaluate the uptake and the toxicity of various ligands in order to mitigate the toxicity of the functionalized nanoparticles.

A common ligand used frequently in order to promote different facets during the growth of the nanoparticles into various shapes is cetyltrimethylammonium bromide (CTAB). It has been demonstrated that, in normal cell lines, this capping agent is extremely toxic and can generate holes in the cell membrane (Leonov *et al.*, 2008). On the contrary, polyvinylpyrrolidone (PVP) has been demonstrated to promote cubic nanoparticles by capping the nanoparticles on {100} facet and reducing the toxicity of silver nanoparticles (Tao, Habas, and Yang, 2008).

3.2 Size and Morphology

Tailoring the size and morphology of the nanoparticles' optical and electronic properties is crucial

for many applications. For example, it has been shown that sharp-edged nanoparticles are suitable for sensor applications based on the increase in their electric field enhancement (Kelly *et al.*, 2003). The most frequently studied shapes are spheres, rods, cubes, nanowires, nanochains, and prisms, and it is believed that they tend to grow to the structure of the Platonic solids (Kim *et al.*, 2004; Montejano-Carrizales *et al.*, 2006; Barnard and Sternberg, 2007). In addition, non-platonic structures have been synthesized, such as nanorods, triangular, and hexagonal platelets (Kirkland *et al.*, 1990; Jin *et al.*, 2003; Germain *et al.*, 2003; Lofton and Sigmund, 2005) as well as elaborate geometries such as nanocages (Oldenburg *et al.*, 1998) and nanostars and other shapes (Chen *et al.*, 2005; Yamamoto *et al.*, 2005; Nehl, Liao, and Hafner, 2006).

Thus, there are challenges in the synthesis of novel nanostructures with tunable structures and functions. One example concerns semiconductor QD nanoparticles (CdO or CdS). Their photoemission can be tuned by varying their size and shape (Jin *et al.*, 2001). In the case of noble nanoparticles, changes in shape make it possible to tune their optical scattering responses: spherical nanoparticles exhibit a single scattering peak compared with anisotropic shapes, such as nanorods and triangles (Buffat, 2003) that exhibit multiple scattering peaks based on highly localized charge polarizations at their corners and edges. By controlling the shape of the nanoparticles, not only can their optical properties be tuned but also the chemical reactivity of their surface. This is directed by the number of edges, sites, bounding facets, and surface-area-to-volume ratio. For example, Pt and Pd nanocrystals of different sizes and shapes are highly selective catalysts (Ahmadi *et al.*, 1996).

Owing to their remarkable properties and a wide range of possible applications, it is essential to control the shape, size, and surface morphology of the nanoparticles. Especially for noble nanoparticles, using colloidal synthesis methods, along with the crystallographic control of the nucleation and growth, results in a variety of architectures. In a typical synthesis, a metal salt is reduced in solution in the presence of a capping agent. The crystallographic control is achieved by tuning the nucleation and the growth steps by using thermodynamic and kinetic parameters. The thermodynamic and kinetic parameters should be controlled simultaneously

in order to obtain the desired shape. The thermodynamic parameters are the reaction temperature, reduction potential, and so on, and the kinetic parameters are the reactant concentration, diffusion, solubility, and reaction rate. Another crucial parameter that governs the shape of nanoparticles is their surface energy. For example, for a material that has an isotropic surface energy, the total surface energy can be lowered by simply decreasing the amount of surface area in a given volume. In this case, the resulting nanoparticle is a perfectly symmetric sphere. In the case of anisotropic materials, the free energy is minimized by nanoparticles' fusion by the low index plane. Theoretical results predicted that, in the case of Cu, Ni, Pd, Pt, Ag, and Au at zero temperature, the high index crystal plane will fuse into linear combinations of low index planes {111}, {100}, and {110} (Frenken and Stoltze, 1999).

Despite the benefits that these properties confer, they can also cause several adverse effects: greater chemical reactivity can cause reactive oxygen species (ROS) production, affect cell morphology, deregulate signaling pathways, and even result in cellular toxicity and cell death. Because of their size, nanoparticles can reach places that other molecules cannot enter. For example, they can reach the nucleus of a cell, and, *in vivo*, nanoparticles can cross placental barriers and accumulate in the mice pups (Gu *et al.*, 2009; Chu *et al.*, 2010).

3.3 Mechanisms of Nanoparticle Toxicity

3.3.1 Reactive Oxygen Species

This is a quite common form of toxicity that occurs when cells are exposed to environmental stress caused by the presence of nanoparticles. ROS can be divided into radical ROS (nitric oxide and hydroxide radicals) and nonradical ROS (hydrogen peroxide). Cells possess a defense mechanism through the glutathione redox system; however, when the concentration of the ROS is too high, it induces various negative effects that lead to cell damage (Stroh *et al.*, 2004). The massive amount of ROS caused by exposure to different nanoparticles is based on their surface-oxidizing capabilities. The mechanism through which nanoparticles generate ROS is as follows: (i) exposure of the cells to an acidic environment (Stroh *et al.*, 2004; Jain *et al.*, 2008; Soto, Garza, and Murr, 2007); (ii) the interaction of nanoparticles with various organelles that

affect their functions (Soto, Garza, and Murr, 2007); (iii) the interaction of nanoparticles with different redox proteins, for example, NADPH oxidase (Pisanic *et al.*, 2009); and (iv) or interaction with different cell surface receptors that can deregulate the cells' signaling pathways (Arbab *et al.*, 2003).

In the case of IONPs, the generation of ROS is typically a transient effect: high concentrations of ROS are found in the first 24 h with a decrease to near control level over 72 h (Arbab *et al.*, 2003). The surface ligand plays a critical role in determining the levels of ROS; for example, when using citrate-coated nanoparticles, a high concentration of ROS was produced in only 4 h of exposure—as opposed to other ligands, such as dextran or lipids, which resulted in the production of a maximum ROS concentration in 24 h (Soenen *et al.*, 2010a). The surface area of the nanoparticles and the stability of the coatings determine the ROS-induced toxicity. In contrast with this study, Gao *et al.* (2007) have shown that IONPs diminish the levels of ROS providing that they are stable and their surface coating does not degrade. Another group, Huang *et al.* (2009), has reported that dextran-stabilized iron nanoparticles sustain cellular proliferation and do not cause any cellular damage. These studies demonstrate that the generation of ROS is a complex subject, and the potential problems associated with cell–nanoparticle exposure along with the inductive mechanisms are still unclear. (Nel *et al.*, 2009)

ROS production has been associated with the toxicity of many other nanoparticles, including QDs, carbon nanotubes, and other metal oxides. The mechanism through which the ROS are produced is generally through the release of M^+ ions or by accumulation in organelles, such as mitochondria (Li *et al.*, 2009). In the case of QDs, a mechanism that can generate ROS other than release of $CD2^+$ ions is the induction of photo-oxidative processes that involve the transfer of the electrons for the excited Fermi level to the oxygen atoms (Li *et al.*, 2009).

For GNPs, ROS generation is unclear. Owing to the fact that gold is an inert metal, GNPs were long considered nontoxic (Connor *et al.*, 2005). However, Li *et al.* (2010) have indicated an increase in the presence of ROS in human lung fibroblasts cultures, their findings were later confirmed by Qiu *et al.* (2010) who demonstrated mitochondrial membrane rupture when human breast adenocarcinoma

cells, MCF-7 cells, were incubated with GNPs. As ROS-induced cellular death is the most common toxicity resulting from nanoparticle exposure, accurate methods to evaluate ROS levels are of crucial importance. The most common method for assessing ROS levels involves dichlorodihydrofluorescein and its derivatives.

3.3.2 Cell Morphology and Cytoskeletal Defects

Owing to the accumulation of nanoparticles of different sizes in various regions within cells, their morphology and cytoskeletal network (Soenen *et al.*, 2009, 2010b) can be affected. Various groups have studied the effects of cellular internalization on the cytoskeletal network (Gupta and Gupta, 2005; Gupta and Curtis, 2004). Here, the coating and the size of the nanoparticles are two important parameters that should be considered. Wu *et al.* (2010) have observed that IONPs induce cytoskeletal defects, such as the disruption of the actin fibers and tubulin networks, when they accumulate in human umbilical vein endothelial cells (HUVECs). Another group has found that IONPs induce cell elongation and an increase in actin stress fiber formation (Soenen *et al.*, 2009). Soenen *et al.* (2010b) have found that lipid-coated nanoparticles cause cytoskeletal disorganization (Soenen *et al.*, 2010b) and dextran- and citrate-coated nanoparticles induce deformation of the actin and tubulin networks.

For QDs, significant cell morphology and structural changes in the actin and tubulin networks were reported by Mahto *et al.* (2010) when 3T3 fibroblasts were incubated with CdSe/ZnSe QDs. GNPs have also been shown to produce cytoskeletal and morphological modifications (Patra *et al.*, 2007). GNPs were described as having a concentration-dependent effect on actin fibers when they were internalized by human dermal fibroblasts. Mironava *et al.* (2010) have further demonstrated that the size of nanoparticles and their concentration significantly affect cytoskeletal filaments, but no sign of toxicity was observed. The effect of different concentrations of nanoparticles on cell morphology and cytoskeletal disruption must be studied in depth in order to find the optimum loading capacity for cell viability. It is important to note that any effect on the cytoskeletal structure may cause secondary disruptions, as it is involved in many signaling pathways.

3.3.3 Intracellular Signaling Pathways and Genotoxicity

Intracellular signaling pathways can be deregulated by disrupting cellular homeostasis using nanoparticles. This deregulation can cause cell death (i) through the genotoxic effects of ROS formation (Singh *et al.*, 2009); furthermore, (ii) owing to their perinuclear localization, cell transcription and translation may occur (Qian *et al.*, 2012), and various proteins and genes can be altered (Kedziorek *et al.*, 2010). The effect of nanoparticles on gene expression and protein altering has not been fully determined; more studies are needed in order to understand changes in the signaling pathways. However, ambiguous results have been reported concerning DNA damage and changes in gene expression levels in the case of IONPs, QDs, GNPs, and SWCNTs/MWCNTs. Several groups have reported no effect on stem cell differentiation, no genotoxicity, and no alteration in gene expression (Kedziorek *et al.*, 2010; Arbab *et al.*, 2005a; Auffan *et al.*, 2006; Zhang *et al.*, 2006; Khan *et al.*, 2007). On the other hand, several other research teams have described inhibition in cell differentiation (Kostura *et al.*, 2004; Chen *et al.*, 2010a) and upregulation in gene expression (Kedziorek *et al.*, 2010) in the case of IONPs. With respect to QDs, based on their high oxidative nature, DNA damage effects such as fragmentation (Hoshino *et al.*, 2004), DNA strand breaks, and activation of p53-associated signaling (Choi, Brown, and Szyf, 2008) can occur. The nanoparticles that have been reported to be the least toxic are GNPs. However, even here, the size of the nanoparticles and their surface chemistry is extremely important. For nanoparticles with a diameter of <3 nm, owing to their capacity to target the nucleus, DNA damage can occur (Gu *et al.*, 2009).

The genotoxic effect of the nanoparticles must be studied in detail before the use of these materials for biological applications. It will be necessary to study gene expression levels in order to evaluate any potential stresses that can upregulate the cell signaling pathways.

3.3.4 Intracellular NP Degradability

It is well known that nanoparticles possess a high surface charge density where the local pH value on the surface can be lower or higher than the value of its surroundings. Moreover, once internalized

by the cell, the nanoparticles will be exposed to a variety of enzymes that are able to degrade almost all bioconjugated ligands (Sealy, 2009). Thus, after the degradation of the ligands, the acidic environment from the endosomes could lead to the oxidative etching of the nanoparticles' surface and, therefore, gradually release free metal ions that potentially affect the cell's homeostasis. IONPs covered with dextran have been shown to degrade in a pH-dependent manner and lead to the release of ferric ions (Arbab *et al.*, 2005b). Similarly, Levy *et al.* (2010) have confirmed this finding. Furthermore, it has been shown that they can cause apoptosis and inflammation (Lunov *et al.*, 2010) and inhibit mesenchymal stem cell (MSC) differentiation (Chen *et al.*, 2010a). For QDs, Gagne *et al.* (2008) have described the destabilization of nanoparticles as time dependent. Cd²⁺ ions have been shown to increase in concentration with an increase in exposure time (Molnar *et al.*, 2010). In addition, their fluorescence decreased as a function of time (Wang *et al.*, 2008).

On the basis of the possible degradation of the ligand and shell, it is important to conduct kinetic studies of the degradation process and quantify the degree of ion release. Furthermore, the ROS formation should be studied if there is any sign of cytotoxicity after degradation.

4 TOXICOLOGY STUDIES OF DIFFERENT CLASSES OF NANOMATERIALS IN NONSTEM AND STEM CELL CULTURES

4.1 Gold Nanocomposites Toxicity in Nonstem Cell Cultures

Among all nanoparticles, GNPs have attracted special attention for the development of biomedical applications because of their surface chemistry and surface stability (Daniel and Astruc, 2004). On the basis of the low toxicity of gold in bulk, it is expected that GNPs will not show any sign of cytotoxicity; however, multiple reports have described damage to cells when they were cultured in the presence of nanosized gold particles (Tarantola *et al.*, 2010; Zhang *et al.*, 2010a; Li, Zhao, and Astruc, 2014).

Unfortunately, a lot of the studies suffer from a lack of standardization; therefore, there is considerable need to stabilize such protocols at different time points and concentrations of nanoparticles. There

is also an urgent need for more nanotoxicological studies, especially studies of different cell types conducted on various key parameters.

The key points in establishing such a nanotoxicology protocol are the size of the nanoparticles (decreasing their size changes the surface chemistry of nanoparticles) and their surface area-to-volume ratio (increasing their surface area increases the reactivity of nanoparticles). Moreover, smaller sized particles can internalize, in particular, subcellular locations. They can penetrate the nuclei of cells and cross the brain barrier resulting in cell deregulation (Simon and Jahnen-Dechent, 2008).

As mentioned previously, another significant factor that may play a role in the cytotoxicity of nanoparticles is the surface coating (during the synthesis either by adsorption of reduction agents or by stabilizing agents). There are different types of surfactants used for the synthesis of GNPs: CTAB, PEG, compounds containing phosphines, and so on. These coatings can either increase or decrease the toxicity of the nanoparticles. For example, using polymer-coated GNPs, the cytotoxicity can be greatly reduced (Alkilany *et al.*, 2009; Chen *et al.*, 2010b).

Early studies suggested that, based on their small size, citrate-GNPs could be internalized by the cells within the first few minutes without any sign of toxicity (Lehmann *et al.*, 2010). However, Thomas and Klibanov (2003) have reported that PEI (polyethylenimine)-modified GNPs induced toxicity in approximately 20% of the cells studied. Similarly, they were able to transfect monkey kidney, having an increase of efficiency by a factor of six compared with PEI alone (Thomas and Klibanov, 2003). Moreover, using dodecyl-PEI complexes, 30% of the cells became apoptotic. Another group, Tkachenko *et al.*, showed that GNPs, alone, and peptide (containing the receptor-mediated endocytosis and nuclear localization signals of HepG2 cells)-functionalized gold nanoparticles are highly compatible. After 12 h of exposure, cell viability was >95% (Tkachenko *et al.*, 2003). The same group, using LDH cytotoxicity, demonstrated the distinct behavior of peptides-BSA-GNP conjugates in different cell lines: HeLa, 3T3/NIH, and HepG2 (Tkachenko *et al.*, 2004). After only 3 h of incubation, 20% cell death was caused in HeLa cells compared with 5% in the 3T3/NIH cell line, using adenovirus fiber protein-BSA-GNPs. This difference in cell viability

may be caused by differences in internalization in different cell lines. When nanoparticle complexes are internalized into the nucleus, they can influence cellular viability as a result of their interaction with DNA.

As reported, surface chemistry plays an important role in the toxicity of nanoparticles. Goodman *et al.* (2004) also tested the toxicity of cationic (ammonium-functionalized) and anionic (carboxylate-functionalized) GNPs in three different cell types: COS-1 cells, red blood cells, and *Escherichia coli* cultures (Goodman *et al.*, 2004). This team found that cationic nanoparticles are more toxic than anionic nanoparticles. However, small statistically insignificant variations were observed between the cell lines.

Similar to the surface coatings, size has been reported to influence the toxicity of nanocomplexes. Connor *et al.* studied the effects in human leukemia cells (K562) of the surface agent and size of the nanoparticles on the uptake and toxicity (Connor *et al.*, 2005). The size of the nanoparticles ranged between 4 and 18 nm and the surface agents tested were biotin, CTAB, cysteine, citrate, and glucose. In all of the cases, the nanoparticles were rapidly internalized by the cells, being clustered into the endosomes. After 3 days of exposure, the biotin- and citrate-modified nanoparticles (C-250nM) were found to be nontoxic, compared with the 90% toxicity of the gold salt (AuCl₄) solution at the same concentration. The CTAB, cysteine, and glucose were reported to not be as biocompatible as citrate and biotin, but no significant statistical difference was found for the 250 nM concentration.

A more detailed study was reported by Shukla *et al.* (2005). They tested the nanoparticles' effect on cell proliferation, on the production of nitric oxide, and ROS in macrophage cells (Shukla *et al.*, 2005). The viability test was conducted after 48 and 72 h, respectively. After 48 h, cell viability was >90%, and there was no sign of proinflammatory cytokines, such as TNF- α and IL-1b. However, after 72 h, the viability decreased to 85%. This decrease in cell viability was not attributed to the nanoparticles, but to the depletion of nutrients from the cell medium, as the medium was not changed for 72 h. In contrast to these studies, Pernodet *et al.* (2006) examined the influence on cell proliferation, the change in cell morphology, and protein synthesis in human dermal fibroblast cells when exposed to GNPs. They reported a decrease in cell number,

which indicates that the nanoparticles were slightly toxic. Furthermore, a progressive accumulation of vacuoles in the cells was observed, which suggests that the internalization of the nanoparticles was not endocytotic but instead occurred through diffusion. Therefore, different mechanisms of internalization could influence the toxicity of the nanoparticles.

Similar to size and surface ligand, the morphology is also an important parameter that influences the cytotoxic behavior of various nanostructures: nanoshells, nanorods, spheric nanoparticles, nanowires, and so on. For example, gold nanoshells have been reported to be nontoxic by Hirsch *et al.* (2003). Although the focus of this study was to show that nanoshells may be used in photothermal ablative therapies, the team also mentioned that the exposure to nanoshells did not cause any cell death. Gold nanoshells are usually composed of a core metal that is coated with a thin shell of gold. These shells confer new properties, such as adsorption and scattering, that can be tuned by varying the thickness of the shell in relation to the core. On the basis of the ability to tune their optical properties, the core-shell nanoparticles are promising materials to be used in medical applications for imaging contrast and photothermal therapy (Lin *et al.*, 2005).

Another group that reported the biocompatibility of gold nanoshells conjugated of anti-HER2 in best cancer cells is Loo *et al.* (2005). Moreover, James *et al.* (2007) studied the *in vivo* biocompatibility of nanoshells in female albino mice (James *et al.*, 2007). Five mice were used for the study. The timescale of the experiment was 28 days. They reported the accumulation of the nanoshell particles in blood in almost all organs. The accumulation in liver, kidneys, spleen, lungs, muscle, brain, and bone was measured, and it was reported that the mice suffered no physiological complications as a result of the presence of the nanoshell nanoparticles. Contradictory to those studies, Su *et al.* (2007) using gold/copper nanoshell incubated in Vero cells for 6 or 24 h, found a decrease in cell viability of 15% at a concentration of 200 mg ml^{-1} after 24 h of incubation. No cytotoxic effect was found at a concentration of 0.001 mg ml^{-1} or in the case of 6 h exposure. The *in vivo* effect of the gold/copper nanoshells was also tested on male BALBc mice for 30 days. It was found that, at a low concentration of 0.001 mg ml^{-1} , no physiological changes were observed, and the viability rates were also found

to be 100% compared; at the highest concentration of 200 mg ml^{-1} , where this team reported a 67% viability.

As in the case of nanoshells, gold nanorods exhibit distinct optical properties because of their transverse and longitudinal Plasmon that can be used in a wide range of biomedical applications. Takahashi *et al.* (2006) found the nanorods to be biocompatible and no physiological complications due to the exposure of skin and muscle to nanorods were observed.

The chemicals that are involved in the process of synthesizing the nanoparticles are very important and direct not only the properties of the nanomaterial but also its toxicity. For example, Niidome *et al.* (2006) showed that the toxicity of CTAB-stabilized PEG-modified gold nanorods on HeLa cells is given by the presence of CTAB. They demonstrated that, when the excess of CTAB was removed from the nanoparticles, the cell viability was 90%.

Different surfactants also direct the internalization potential of nanoparticles. For example, Huff *et al.* have shown that gold nanorods functionalized with CYAB and mPEG-DTC-coated are internalized differently by KB cells (Huff *et al.*, 2007). They demonstrated that CTAB-gold nanorods internalized progressively in the perinuclear region of the cells after 5 days, compared with the nanorods functionalized with mPEG-DTC that inhibited the internalization of the nanoparticles.

Therefore, in order to fulfill a proposed goal, it is important to carefully choose the synthesis chemicals. Furthermore, standardized incubation conditions along with a standard method of characterizing the nanoparticles and large-scale comparative studies both *in vitro* and *in vivo* are needed in order to generate sufficient data that will lead to a better understanding of this field.

4.2 Gold Nanocomposite Toxicity in Stem Cell Cultures

Stem cells are attractive tools for future medicine. They have the potential to differentiate into different lineages, from cardiac to neuronal to fibroblast, and so on. Our group has previously demonstrated an efficient method to differentiate MSCs using engineered collagen-gold nanoscaffolds for cardiac and neuronal stem cells (Zhang *et al.*, 2008). These findings demonstrated that MSCs can be

used to recuperate lost myocardium or to assist in brain injury repair (Zhang *et al.*, 2010b; Asahara *et al.*, 1997; Valarmathi *et al.*, 2009; Al-Khalid *et al.*, 2003). However, the exact mechanism of the vascular repair of lost tissues or organs is unclear. A few approaches have been reported, such as tracking the prelabeled cells *in vivo* and performing histological studies at various time points (Lee, Dennis, and Gerson, 2008). The cell labeling should be performed with efficient contrast agents that have long-term stability, sensitivity, and are nontoxic. Traditional agents are radionuclides. The disadvantage of these is that they have a short half-life (Sheikh and Wu, 2006); as a result, they are not efficient for long-term imaging.

By taking advantage of the benefits of nanotechnology, the construction of artificial organs and tissues becomes more promising. Before the regeneration of a specific organ, using nanoparticles, the specific stem cells can be obtained *in vitro* through differentiation, and, during transplantation, the cell can be tracked. For tracking, nanoparticles are superior to radionuclides because they may be used over the long term (Frangioni and Hajjar, 2004; Huang *et al.*, 2009; Guzman *et al.*, 2007; Ferreira, 2009). In addition, owing to their optical properties, photoacoustic imaging can be performed *in vivo* (Brooking, Davis, and Illum, 2001).

Although nanotechnology makes it possible to control the micro- and nanoenvironment of the stem cells, this support and regulation can have harmful side effects. Complex studies that assess their toxicological effects should be conducted. There is relative lack of information concerning the impact of these materials on stem cell biology. As nanomaterials can penetrate the biological membranes, they can affect the physiology of the cell (Brooking, Davis, and Illum, 2001). It is urgently needed to evaluate their effects on stem cell self-renewal and differentiation. However, at this time, data concerning the toxicological effects of nanomaterials on stem cells are limited. We will therefore summarize and discuss the toxicological effects of different nanoparticles and nanomaterials on MSC cultures.

Ricles *et al.* (2011) measured the MSCs' viability and toxicity when they were in contact with different sized ligand-GNPs as follows: 20, 40, and 60 nm stabilized with citrate (CS) and poly-L-lysine (PLL) (Figure 2a–f). The viability studies were assessed

using 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) assay and LIVE/DEAD stain assay at various time points (1, 7, and 14 days) for a period of 2 weeks. The results given by the LIVE/DEAD along with the MTT assay are shown in Figure 2. For the LIVE/DEAD assay, the green cells were considered to be alive, and cells colored in red were considered dead (Figure 2e). The controls used for comparison were MSCs cultivated without nanoparticles. According to the microscopy study, cell death was minimal. (The dead cells are indicated by white arrows.)

Using the MTT cytotoxicity assays shown in Figure 2f, the cell viability was measured at 1, 4, and 7 days after incubation with the nanoparticles. The control contain cells without nanoparticles. The results were statistically compared using a $p < 0.01$. A lower cytotoxicity was observed in the case of citrate-GNPs of 20- and 60-nm size. In addition, after 7 days of incubation, the 60-nm-stabilized PPL presents a statistically significant toxicity compared with the control. However, after 4 days of exposure to all nanoparticles systems, the MSCs present a higher proliferation and growth than the control.

Using lysine and aspartate as a capping agent and collagen as a template, we were able to analyze the toxicity of GNPs of different shapes, nanochains, nanowires, bares, and triangular wires, on placental-derived MSCs. The morphology of these nanostructures is shown in Figure 3a.

The interaction of those nanostructures with MSCs along with their effect was assessed by microscopy and MTT assays (Orza *et al.*, 2013). The incubation time at which MTT assays were performed was 1.5 h and, respectively, 4 days. The phase contrast microscopy in white light and fluorescence microscopy using filter for 488-nm excitation images are shown in Figure 3b.

The internalization of the nanoparticles takes place within the first 20 min. After 1.5 h of exposure, the phase contrast microscopy revealed a progressive accumulation of the nanoparticles in the perinuclear space. After 4 days of exposure to nanoparticles to MSCs culture, this accumulates in the cytoplasmic area. No sign of toxicity was observed. The MTT assay confirmed an increase in proliferation when cells were cultivated in the presence of nanostructures, with statistical significance in the case of the gold-collagen nanostructure (Figure 3c).

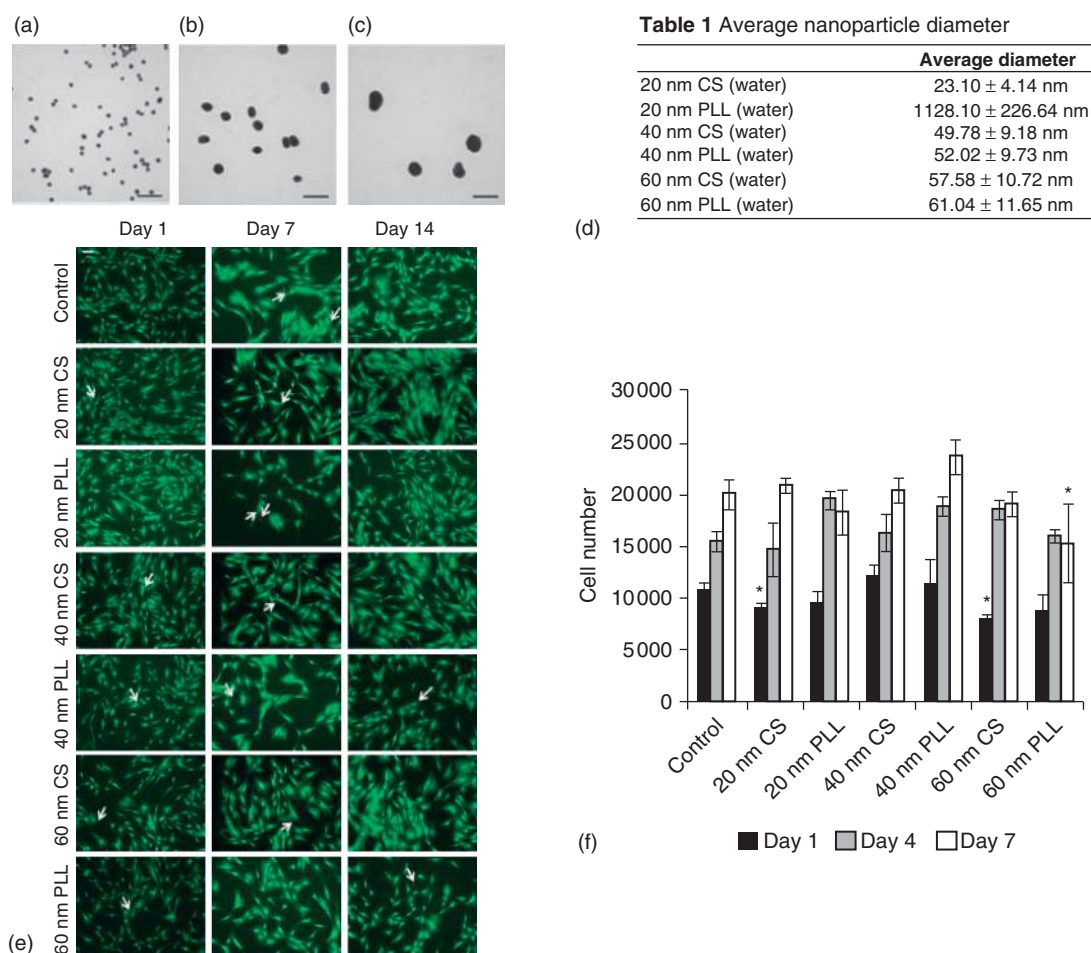


Figure 2. (a–f) TEM images of gold nanoparticles (a, b, c) along with their size average, (d) live/dead of the cell, and (e) Mtt proliferation assay after 24 h of incubation with the nanoparticles. [Rickles *et al.* (2011) with permission from John Wiley & Sons, Inc.]

4.3 Super-Paramagnetic Iron Oxide Nanoparticles (SPIONs) in Nonstem and Stem Cell Cultures

IONPs are another class of potential nanostructures that have shown good biocompatibility with stem cells, being capable of metabolizing and sustaining cell proliferation and growth without affecting the cell phenotype or its differentiation capability (Li *et al.*, 2008; Wang *et al.*, 2009; Yang *et al.*, 2011; Crabbe *et al.*, 2010; van Buul *et al.*, 2011)

The literature reports a variety of studies that employed iron nanoparticles for stem cell labeling and tracking using magnetic resonance imaging (So *et al.*, 2010; Zhou *et al.*, 2010). Some toxic effects have been reported in the case of the naked

nanoparticles. Coated nanoparticles have been found to be relatively biocompatible. Gupta *et al.* showed that PEG-coated nanoparticles are nontoxic in a dose of up to 250 mg ml⁻¹ as more than 99% of the cells remained viable (Gupta and Wells, 2004). To the contrary, bare iron oxide incubated on fibroblast culture induced 25–50% cell death. In a more detailed study, Gupta *et al.* examined the influence of the nanoparticles' concentration on their toxicity. The team used the concentrations of 0.05 and 2.0 mg ml⁻¹, which resulted in cell death of 20 and 60%, respectively. However, Yu *et al.* found that PMAO-PEG-coated SPIONs were nontoxic, as cell viability was decreased by only 9% (Soenen *et al.*, 2010a). Similarly, Hussain *et al.* have found that bare IONPs are nontoxic up to a dose of

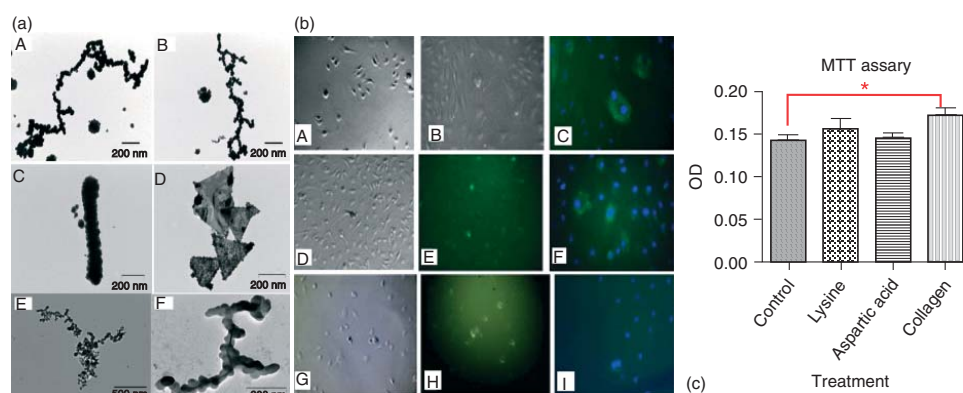


Figure 3. a. (A–D) TEM images of one-dimensional metallic nanostructures formed from lysine-capped gold (A, B), aspartate-capped gold (C, D), and collagen-template (E, F)-inverted phase microscopy images ($\times 40$, PlasDIC): top panel: (A) after 1.5 h, (B) after 4 days of cultivation in the presence of GNP aspartate, and (C) fluorescence microscopy images (filter 488 nm) of fixed MSC cells after 4 days of exposure to GNP-aspartate (counterstaining with DAPI); middle panel: (D) phase contrast image of placental derived MSC after 1.5 h of cultivation in the presence of GNP-collagen, (E) fluorescence image (filter 488 nm) of MSC cells after 1.5 h of exposure to GNP-collagen; (F) immunostaining with collagen FITC antibody (488 nm) of fixed MSC cells after 4 days of exposure to GNP-collagen (counterstaining with DAPI); bottom panel: (G) phase contrast images of placental derived MSC after 1.5 h of cultivation in the presence of GNP-lysine; (H) fluorescence image (filter 488 nm) of MSC cells after 1.5 h of exposure to GNP-lysine. (I) Fluorescence image (488 nm) of fixed MSC cells after 4 days of exposure to GNP-lysine (counterstaining with DAPI). Proliferation assay for mesenchymal stem cells in the presence of nanostructures. [Anamaria Orza *et al.* (2013). Reproduced with permission from Taylor and Francis.]

250 mg ml⁻¹ (Yu *et al.*, 2006). In addition, several other groups have reported that PEG-coated IONPs are nontoxic. As mentioned previously, surface ligands play an important role in the toxicity. For example, Wan *et al.* have demonstrated the effect of three different coatings: MPEG-Asp3-NH₂-, MPEG-PAA-, and PAA-coated IONPs. The first exhibited no toxicity (Wan *et al.*, 2007) in contrast with the other two coatings, which reduced cell viability by 16% at a nanoparticle concentration of 400 mg ml⁻¹. The toxicity of IONPs was linked with both ROS production and the mechanism of the cellular uptake. Hu *et al.* found that pristine iron nanoparticles generate a decrease in cell viability of 90% after 5 days of incubation. However, PEGMA-coated nanoparticles were nontoxic (Hu *et al.*, 2006).

Chang *et al.* (2012) have shown that MSCs incubated with IONPs are not affected by the presence of the nanoparticles. More than 95% of the cells were viable with concentrations of 25, 50, 75, and 100 mg ml⁻¹. Moreover, the morphology of the cells was not changed even with a high accumulation of nanoparticles (Figure 4a-A). This team also reported that a concentration of 50 mg ml⁻¹ is the most efficient dose for the uptake. In addition, the linear correlation between cell density and relaxation values of T1WI (r₁, R₂ 1/4 0.9922) and T2WI (r₂, R₂ 1/4 0.9982) was obtained. TEM gives information about the mechanism of nanoparticle uptake. Initially, the nanoparticles were found in the endocytotic cups at the cell surface, which suggests the initiation of the uptake through endocytosis (Figure 4b-A). The nanoparticles were also found accumulated in the cytoplasm. In order to confirm the endocytosis uptake, colchicine was used to induce depolymerization of the microtubules. A reduced content of nanoparticles was observed [analysis of variance (ANOVA), *p* < 0.05; Figure 4b-B]. Moreover, the nanoparticles induced greater cell proliferation compared with the cell viability of the control (cells cultured without nanoparticles) (one-way ANOVA, *p* < 0.05; Figure 4B). Figure 4A,B shows the promotion of MSC proliferation given by analyzing the PD levels with a slope of 0.54 for MSCs cultured in the presence of the nanoparticles and 0.35 for MSCs cultured without nanoparticles. Furthermore, when using nanoparticles, performing the chi-square test (Figure 4C), a considerable increase in cell numbers in the S and G2/M phases was observed.

4.4 Semiconductor Nanoparticles in Nonstem and Stem Cell Cultures

Semiconductor nanoparticles are usually extremely toxic based on their elemental composition; however, despite the potential health risks, their properties make them ideal candidates for a wide range of biomedical application, such as new drug delivery or biomedical imaging agents. The advantage of using semiconductor QDs as imaging tools is based on their size-property tunability. By simply changing the size of the nanoparticles, the fluorescence emission peak can be shifted to a broad range of wavelengths. This property is necessary for biological applications because, due to the existence of the endogenous fluorophores, that can emit fluorescent peaks similar to the small contrast agents and finally block their signal.

Here, we focus on the current research on QD toxicity. As in the case of other metal nanoparticles, QDs can be synthesized in a variety of sizes and shapes and have different types of surface coatings or functional groups. However, for biological applications, QDs are usually composed of core-shell structures containing atoms from group II–IV or II–V. Examples of QDs that have been synthesized are as follows: GaAs, GaN, InP, CdTe, CdS, PbSe, ZnS, and so on (Chan *et al.*, 2002; Liu *et al.*, 2014). These metals are known to be extremely toxic even in small concentrations. Degradation of the core QD can occur in an oxidative environment, leading to the release of metal ions and finally causing acute toxicity. Therefore, the synthesis procedure should be well controlled in order to prevent core degradation. The most versatile QDs for biomedical application are believed to be CdSe/ZnS; thus, most toxicity studies have been focused on this material (Portney and Ozkan, 2006; Hardman, 2006).

Akerman *et al.* (2002) have studied *in vivo* using normal BALBc mice the toxicity and tissue distribution of CdSe/ZnS QDs coated with peptides (GFE, F3, and LyP-1). They reported that, in addition to the targeted tissue, the QDs accumulated in the liver and spleen. However, by adding PEG on the surface of the QDs, the nonspecific accumulation was reduced. In addition, the group reported no sign of acute toxicity (Akerman *et al.*, 2002). Another group that obtained the same result is Larson *et al.* (2003). No severe effects were observed after imaging mice that had been injected with CdSe/ZnS QDs. The team hypothesized that the QD coating was very stable

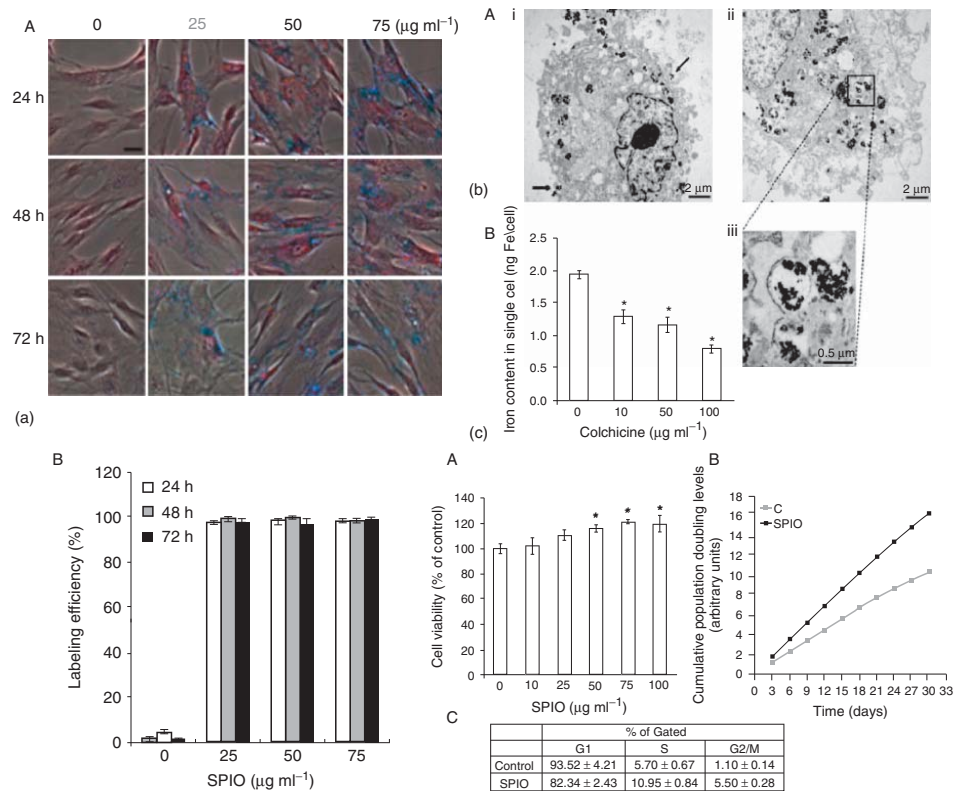


Figure 4. (a-A) Optical images of the internalization of iron oxide nanoparticles of 0, 25, 50, and 75 mg ml^{-1} in mesenchymal stem cells for 24, 48, and 72 h using Prussian Blue. (a-B) The labeling efficiency, 95%, at different concentrations for 24, 48, and 72 h. (b-A) TEM images showing the intracellular distribution of iron oxide nanoparticles after 24 h of incubation. (b-B) Calculated iron content of a single cell after 24 h of incubation. (c-A) MSCs' viability after the cells were incubated with 50 mg ml^{-1} of SPIONs for 24 h. (c-B) Calculation of the doubling level of the MSCs based on the results obtained by the proliferation assay test. (c-C) Flow cytometry data showing the biocompatibility of the nanoparticles; the number of cells increased when they were cultivated in the presence of the nanoparticles. [Seleverstov, O *et al.* (2006). Reproduced with permission from the American Chemical Society.]

and that the nanoparticles were eliminated from the body before the coating began to degrade.

Surface chemistry plays a primary role in determining the final property of the QDs. The effects of different surface coatings were tested *in vivo* by Ballou *et al.* (2004). The QDs were functionalized with poly(acrylic acid), mPEG-750, mPEG-5000, and COOH-PEG-3400. The mPEG-5000-coated nanoparticles were distributed in the liver, spleen, and bone marrow. Among all of the coatings, mPEG-750 was found to be the optimum ligand for reducing nonspecific accumulation. A long-term study of m-PEG-750 revealed that the QDs remained in the liver, lymph nodes, and bone marrow (Ballou *et al.*, 2004).

Similarly, Fischer *et al.* (2006) have studied the toxicity and the distribution of CdSe/ZnS QDs coated with BSA and lysine. They observed that more than 40% of the lysine-QDs were taken up by the liver and 99% of the BSA-QDs were taken up after 90 min. In conformity with other reported studies, small amounts of QDs were found in the spleen, kidney, and bone marrow. The group also measured the size of the QDs after internalization and found that they retained their size after 90 min of exposure (Fischer *et al.*, 2006). The previous literature reports revealed that QDs are relatively nontoxic, as, after injection, the animals did not show any sign of abnormal behavior.

However, CdO₂ is known to be an extremely toxic material that can induce cell damage through mitochondrial and oxidative stress. When the QDs' surface is functionalized with a nontoxic ligand, the ligand protects the surface from undergoing oxidation. The cadmium ions remain bonded on the ZnS atoms; thus, no free ions are released, and they will show relatively little sign of toxicity. This hypothesis investigated by including an additional ZnS shell on the CdSe surface. The results showed that the oxidative degradation of CdSe nanoparticles due to their exposure to air was significantly reduced and the toxicity was lowered (Kirchner *et al.*, 2005; Chan, Shiao, and Lu, 2006).

The mechanism of toxicity of bare nanoparticles, CdSe QD, was tested by Chan *et al.* The group found that cellular death was induced by apoptosis by activating Jun N-terminal kinase (JNK) in a dose-dependent manner. An increase in Bax and Bcl-2 protein was observed based on activation of caspases 9 and 3 (Chan, Shiao, and Lu, 2006). Various studies have been reported concerning the

effect of additional surface coatings on bare QDs. The functionalization of QDs has to be performed in such a way as to guarantee that they will remain stable. The surface manipulation reaction can be conducted by direct synthesis on the surface of the QD, ligand exchange reaction, or coupling reaction. The ligand chosen should be biocompatible, water soluble, and provide good stability. Shiohara *et al.* have studied the toxicity of mercapto-undecanoic acid (MUA)-coated CdSe/ZnS QDs (520-, 570-, and 640-nm emission) on three different cell lines: Vero cells, Hela cells, and human primary hepatocytes. Different concentrations of QDs (from 0 to 0.4 mg ml⁻¹) were incubated for over 24 h. It was observed that, by increasing the concentration of the QDs, the viability of the cells decreased. Moreover, the group reported that the coating increases the toxicity of the nanoparticles (Chan and Nie, 1998).

Similarly, Hoshino *et al.* (2004) studied the influence of toxicity provided by the surface coating. Three different coatings were tested: MUA, cystamine, and thioglycerol. As in the study reported by Shiohara *et al.* (2004), MUA caused more toxicity than the nonfunctionalized QDs. Thioglycerol seemed to be the only coating that promoted higher cell viability. Other methods to decrease toxicity include the use of amphiphilic polymers. For example, Duan and Nie (2007) tested the toxicity of PEI-coated QDs in HeLa cells. They found that the system is toxic; however, by grafting the surface with PEG, the system's toxicity was reduced. They also reported that the distribution inside the cells appeared to have changed: the system PEI-g-PEG4 QDs accumulated in the perinuclear region and showed good cell viability, whereas the PEI-g-PEG2 QDs accumulated at a high concentration in the cytoplasm and had significant toxicity (Duan and Nie, 2007).

The PEG precursor seems to be suitable both to achieve stability and to decrease the toxicity of the QDs. Ryman-Rasmussen and Riviere (2007) tested three different PEG precursors-QDs (PEG, PEG-amine, and carboxylic acids). They found that they are localized in cytoplasm, the perinuclear region of the cell, and the nucleolus. After 24 h of incubation, no sign of toxicity was observed.

Other important factors that can affect the toxicity of QDs are their size and the concentration. Nanoparticles of smaller size and in higher concentrations have been reported to be the most toxic. This finding can be attributed to the small

area-to-volume ratio of the smaller nanoparticles (Shiohara *et al.*, 2004). For example, Kirchner *et al.* (2005) tested the cytotoxicity of CdSe/ZnS QDs in four different cell lines: normal rat kidney (NRK) fibroblasts, MDA-MB-435S breast cancer cells, Chinese hamster ovary (CHO) cells, and rat basophilic leukemia (RBL) cells. After 18 h of exposing the cells to the QDs, the lowest percentage of cell viability was obtained using the smaller sized nanoparticles. Similarly, Hsieh *et al.* (2006) have reported size-dependent toxicity. Another parameter that should be well established in order to control the toxicity of the QDs is their concentration. The same group reported that the concentration influences the delivery into the cell. For example, a 15 nM concentration is efficiently internalized by the cells. However, a 10-fold increase in the concentration resulted poor uptake. It should be noted that this finding contradicts other reports. Other research groups have found that the uptake improves with increasing concentrations (Kirchner *et al.*, 2005; Shiohara *et al.*, 2004; Ryman-Rasmussen and Riviere, 2007; Amna *et al.*, 2013).

Seleverstov *et al.* (2006) first showed a size-dependent uptake of QDs that was autophagy mediated. QD525 and QD605 were used to explore the suitability of those nanoparticles for stem cell labeling. They showed a different distribution inside the cells and also good biocompatibility with the MSCs. Two different concentrations were tested: 5 and 10 mM. The incubation time was 24 and 48 h, respectively. No significant differences in distribution were found after 24 h (Figure 5a (A–E)). A large amount of nanoparticles was found in the perinuclear region of the cells. However, many of the QDs were found accumulated in the endosomes (Figure 5a (A)), lysosomes, and with mitochondria. After 48 h, the two sizes of nanoparticles were distributed differently in the cells. For the QD525, the majority, which had initially been found in the cytoplasm, had migrated into the cytosol; many others were found in mitochondria and Golgi cisterns (Figure 5a (C)). In addition, approximately 2% of the cells were found to be dead (Figure 5a (D)). However, in the case of QD605, for both 24 and 48 h of incubation time, the particles were agglomerated in high concentrations in the cytosol and a few in lysosomes (Figure 5a (E, F)). Cytotoxicity, metabolic, and proliferative assays were also performed, and no significant differences were found (Figure 5c–f).

4.5 Carbon Nanomaterials in Nonstem and Stem Cell Cultures

Carbon materials constitute a new class of nanomaterials that is promising for a wide range of applications (Bianco *et al.*, 2005; Sinha and Yeow, 2005; Kohli and Martin, 2005). The uniqueness of these nanomaterials is based on their physicochemical properties, which confer them distinctive thermal/chemical stability, high conductivity, and special mechanical properties. In the literature, there are a variety of publications that report the benefits of carbon materials for biomedical application: tissues engineering, drug delivery, fluorescent contrast agents, and so on (Bianco *et al.*, 2005; Sinha and Yeow, 2005; Kohli and Martin, 2005). The most important parameters that have considerable impact on their toxicology are similar to those of other metal nanomaterials: size, shape, surface charge, method of synthesis, chemical composition, and, not the least, surface chemistry and stability.

Several studies have been focused on studying the biocompatibility of these materials under different experimental conditions and on various cell types (Panessa-Warren *et al.*, 2006; Smart *et al.*, 2006). Shvedova *et al.* (2003; Akhavan, Ghaderi, and Akhavan, 2012; Jastrzębska, Kurtycz, and Olszyna, 2012; Seabra *et al.*, 2014) have shown that increasing the concentration and the incubation time of SWCNTs in human epidermal keratinocyte cultures increases oxidative stress, and mitochondrial changes were observed. They also noted that, by using metal chelator, the toxicity can be reduced, thus suggesting that the catalyst used in the synthesis of the nanomaterial plays a determining role in inducing the final toxicity. Moreover, Jia *et al.* (2005) found that even the lowest dose of SWCNTs, 1.41 mg cm⁻², induced more than 20% cellular death.

Another parameter that has been suggested as being responsible for nanotubes' toxicity is their aggregation. Wick *et al.* (2007) studied the influence of the agglomeration SWCNTs' toxicity. Four types of SWCNTs—graphite and agglomerated SWCNTs resulting from the synthesis, as well as SWCNT bundles and pellets produced by centrifugation of the agglomerated SWCNTs—were tested (Free, Shaw, and Levy, 2009). The results showed that the nonagglomerated SWCNTs, pellets, did not induce adverse toxicity. However, contradictory studies, such as the reports of Tian *et al.* (2006),

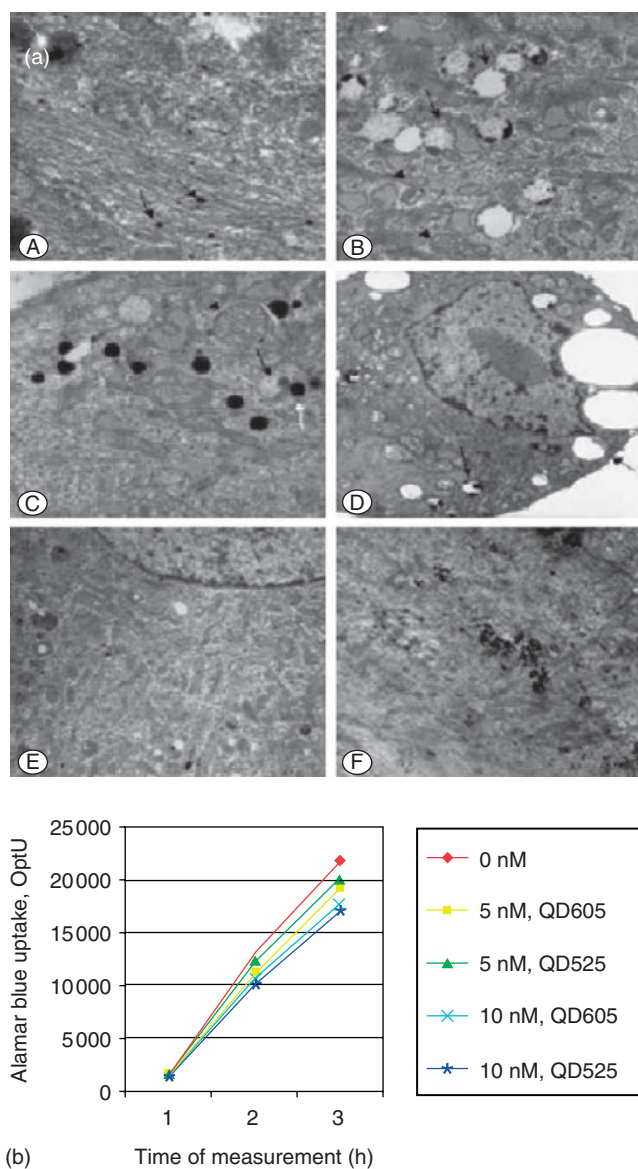


Figure 5. TEM images of (A) QD525, 24 h of incubation, the nanoparticles were found to be taken up into endosomes and lysosomes, magnification 30 000; (B) QD525, 72 h after incubation, the nanoparticles were found in large vacuoles, endoplasmatic reticulum, and destroyed mitochondria, magnification 7000; (C) QD525, 72 h after incubation, nanoparticles were found in vacuoles, the cell mitochondria, and the endoplasmatic reticulum was damaged, magnification 12 000 $\times$, magnification 3500; (D) QD525, 72 h after incubation, the cell is damaged, and the nanoparticles were found in vacuoles, magnification 3500 $\times$; (E) QD605, 24 h after incubation, they were found in the cytoplasm and a few lysosomes, magnification 7000; (F) QD605, 72 h after incubation, no significant changes from 24 h incubation, magnification 12 000 $\times$. (b) Alamar blue metabolic assay. (c) LDH cytotoxic/membrane stability assay. (d) Proliferative assay via BrdU incorporation. (e) MTT metabolic/proliferative assay. [Chor Yong Tay *et al.* (2010). Reproduced with permission from Elsevier.]

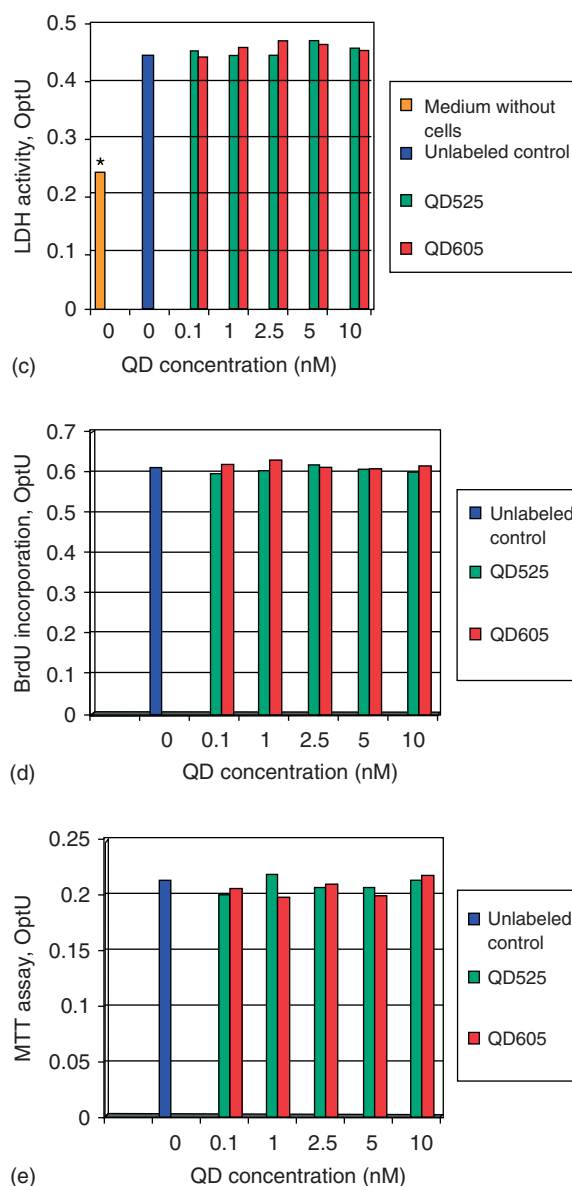


Figure 5. (Continued)

have been published. These two groups used two different cell lines for testing the biocompatibility: asbestos-induced lung-cancer cells versus keratinocytes. Thus, this explains the conflicting results. However, these contradictory results are still questionable, and more standardized studies, for example, using the same cell lines, should be performed in order to come to a conclusion concerning the SWCNTs' aggregation.

Another form of carbon nanomaterial is the MWCNT. Their biocompatibility results are similar to those of SWCNTs. A dose- and time-dependent toxicity has been reported by Monteiro-Riviere and Inman (2006) who demonstrated an increase in MWCNT accumulation from 59% at 24 h to 84% after 48 h. A decrease in cell viability was observed that was coupled with the increase in the concentration. A comparison of these results with

those published by Shvedova in connection with SWCNTs suggests that the nanotubes are toxic. In the case of Shvedova *et al.* (2003), the toxicity was associated also with the presence of the catalyst in the solution. Monteiro-Riviere and Inman (2006) suggest that the cytotoxicity of the nanotubes may be associated with the attachment of the nanotubes to the cell membrane surface and subsequent internalization, as they were found to be near the nucleolus of the cells. Dose- and time-dependent toxicity has also been reported by Sato *et al.* (2005) and Bottini *et al.* (2006).

In addition to the concentration, the surface coating affects toxicity; for example, hydrophobic MWCNTs have been reported to be less toxic (Magrez *et al.*, 2006). However, by diluting the MWCNTs solution, Flahaut *et al.* found a decrease in cell viability of HUVECs (Flahaut *et al.*, 2006). They concluded that the nanotubes were not toxic as the viability of the cells was greater than 75%. The group suggested that this was a result of the nanotubes' agglomeration. By exposure of tissue cultures to MWCNTs, an inflammatory response has been reported (Ding *et al.*, 2005). Ding *et al.* (2005) showed that exposure to high concentrations induces immune and inflammatory gene overexpression. Moreover, other groups have observed changes in protein expression, irritation, and finally cell apoptosis (Witzmann and Monteiro-Riviere, 2006).

From what we know at the present, the properties that make nanoparticles unique are their size, surface area, composition, solubility, and morphology. However, these properties could simultaneously be responsible for their potential danger to human health. All of these parameters are in a strong relationship with their biocompatibility. For example, the size and the concentration are responsible for the cytotoxicity of the nanotubes. Thus, choosing the right method of synthesis and other parameters could lead to potentially biocompatible nanomaterials that could be useful for a variety of bioapplications. Nevertheless, the biocompatibility of carbon materials tested on stem cell cultures has not been extensively studied. Some groups report good biocompatibility (Hirsch *et al.*, 2003; Sayes *et al.*, 2004; Loo *et al.*, 2004; Muller *et al.*, 2005; Uo *et al.*, 2005; Haslam, Wyatt, and Kitos, 2000; Mosmann, 1983; Cunha *et al.*, 2012; Gupta *et al.*, 2013; Ryoo *et al.*, 2010) and others report contradictory results (Zhu *et al.*, 2007). However, the size of the nanomaterials has

a great impact on their toxicity. For instance, Wang *et al.* (2013b) show a size-dependent toxicity of graphite oxide, such as graphite sheet with a diameter >20 nm is biocompatible with mouse embryonic stem cells, whereas smaller sizes show increased cell toxicity. Zhu *et al.* (2007) have reported the toxicity of MWCNTs by inducing DNA damage in mouse embryonic stem cells, whereas SWCNTs induced the formation of free radicals due to the accelerated oxidative stress (Manna *et al.*, 2005; Monteiro-Riviere *et al.*, 2005).

Tay *et al.* (2010) evaluated the effect of SWCNTs when in contact with human MSCs. They evaluated their effect on cell proliferation, morphology, cytoskeletal networking structures, and gene expression. The diameter and morphology of the SWCNTs used for the experiment are shown in Figure 6a. The diameter of the nanotubes was approximately 50–60 nm. By performing a kit-8 (CCK-8) (Dojindo, Japan) assay that measures the dehydrogenase metabolic activities of the cells, it was observed that the cell proliferation was slower on nanotube films (Figure 6b). Furthermore, after 3 days of incubation, the morphology of cells in the presence of nanotube films had changed: they became more larger and more spread out. As vinculin plays an important role in controlling cell spreading and viability, a vinculin expression assay was preformed. It was found to be homogenously distributed throughout the cell body, compared with control cells in which it was found only at the periphery.

Quantitative analysis has been performed for the vinculin number as shown in Figure 6d. The vinculin number was significantly higher when cells were cultivated in the presence of SWNTs, but their size did not change significantly. Moreover, cell confluence on SWCNT films was significantly higher on days 3 and 7, but, by day 14, the cells on both substrates were confluent because of the myotic cell cycle, and no significant differences were found. In order to have a better understanding on the focal adhesion and cytoskeletal orientation, immunohistochemistry assays were performed for both F-actin and vinculin. No changes were observed for the expression of F-actin cytoskeleton after 24 h; however, after 3 days, the stress fibers became more evident in both groups. Nevertheless, higher stress is observed for cells cultivated on cover slips (control) overall (Figure 6c).

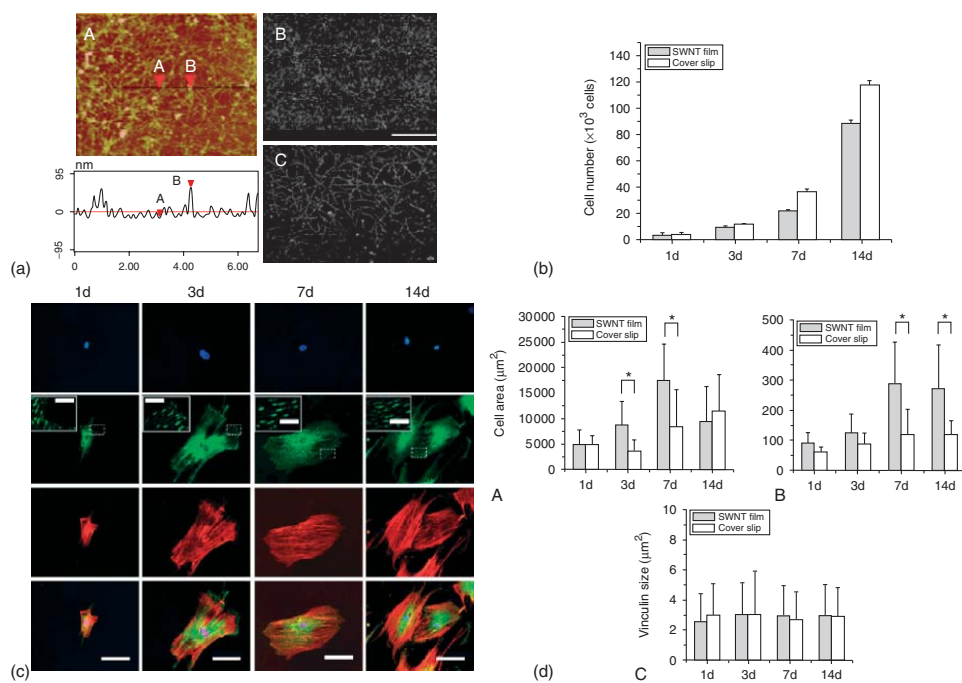


Figure 6. (a) AFM image of the SWCNTs film (A), SEM image of the SWCNTs film magnification 6000 $\times$ (B), 20,000 $\times$ (C). (b) Cell proliferation of the MSCs stem cells cultivated in the presence of the SWCNTs film compared with the control, cells cultivated on the cover slips at predetermined time points. (c) Immunohistochemistry assay for labeled F-actin (red) and vinculin (green), cells cultured on SWCNT-coated cover slips for 1, 3, 7, and 14 days. (d) Statistical analysis of cell spreading area (A), vinculin number (B), and size (C). Vinculin size for the MSCs cultured both in the presence of the nanotube substrate and control at days 1, 3, 7, and 14. [With kind permission from Tay *et al.* (2010).]

5 CONCLUSIONS

In this chapter, we have compared the effects of five types of nanomaterials on tissue cultures for both normal and MSCs. We have demonstrated that the toxicity of nanomaterials is greatly influenced by their composition and physicochemical properties (e.g., size, shape, and surface agent). Furthermore, we have shown that, by choosing the appropriate conditions for the synthesis and stabilization of nanomaterials, their toxicity can be controlled. Nevertheless, there are still doubts as to whether the metal nanoparticles are biocompatible and safe for use in biomedical applications. In order to answer this question, a great deal of research must be performed in order to advance the knowledge in this field. The standardization of toxicological protocols, as well as characterization methods, will be necessary. Moreover, comparative studies using different types of cells with various nanoparticles are crucially needed in order to generate sufficient comparative data.

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Predictive Mechanisms in Stem Cells: An *In Vitro* System-Based Method for Testing Carcinogenicity

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1 INTRODUCTION

Stem-cell-based systems, either derived from human embryonic stem (hES) or induced pluripotent stem (iPS) cells, hold a tremendous promise for applications in regenerative medicine, tissue engineering, and drug discovery because of their inherent capacity to grow indefinitely and to differentiate into all mature cell types of the body (Thomson *et al.*, 1998; Takahashi *et al.*, 2007). These characteristics have led to the development of cellular models with relevance for the human situation *in vivo* in order to reduce the need for experimental animals in testing of drugs, cosmetics, and other chemical compounds (Jensen, Hyllner, and Björquist, 2009).

A major aspect in predictive toxicology is the improvement in the assessment of the carcinogenic hazard (and ultimately the risk) due to the exposure to chemicals. It has been addressed in Europe with the REACH guidelines aiming to assess toxicity of an estimated number of 68 000 chemicals (Hartung and Rovida, 2009). Until now, the majority of tests are based on *in vivo* assays, in particular on the two-year rodent bioassay for carcinogenicity. Besides the challenge of replacing animal testing (it has been estimated that full compliance with REACH legislation for all end points of toxicity will require a grand total of 54 million vertebrate animals

and will cost €9.5 billion over the next decade), it has been argued that the effects of chemical exposure differ widely in rodents and humans, and this leads to a high number of false positive predictions. For example, cholesterol-lowering drugs among many other pharmaceutical agents approved as safe drugs for human use by the FDA (Food and Drug Administration) were classified as rodent carcinogens (Ward, 2008). Thus, it has been understood that human *in vitro* assays must be developed for predicting carcinogenic effects of chemicals in human more reliably (Vinken *et al.*, 2008).

High-throughput technologies such as microarrays have opened the way to a systemic understanding of toxicology and carcinogenicity (Waters and Foster, 2004). Such systems toxicology approaches offer the chance for disclosing valuable mechanistic information on toxic modes of action of substances in the assay system under study, which obviously is promising in view of the urgent need to develop better (*in vitro*) tests for chemical safety. Mechanistic approaches require the analysis of whole-genome data at the pathway level incorporating knowledge on human interaction networks (Kitano, 2002; Wierling, Herwig, and Lehrach, 2007). Pivotal for such approaches is the availability of sufficient information on human pathways, related to cancer initiation and progression, along

with computational approaches that combine results from high-throughput experiments with biological networks (Bader, Cary, and Sander, 2006). One such resource is the ConsensusPathDB, a meta-database of human molecular interactions, which can be used to interpret genome-wide expression data at the pathway level (Kamburov *et al.*, 2013) and, thus, to derive predictive pathway patterns in order to distinguish different toxicity classes.

A fundamental role for future human *in vitro* carcinogenicity testing has been assigned to stem cells and their combination with -omics approaches as well as data integration and bioinformatics tools in order to create a cancer AOP that assists in risk assessment in a reasonable timeframe.

2 GENE EXPRESSION IN STEM-CELL-DERIVED HEPATOCYTES

It has been shown in several studies (Hay *et al.*, 2008; Basma *et al.*, 2009) that hES-cell-derived hepatocytes are able to carry out a range of hepatocyte functions, for example, storage of glycogen and generation and secretion of plasma proteins. More importantly, the cells express several members of cytochrome P450 enzymes capable of converting the substrates to metabolites and respond to chemical stimulation.

The use of hES-cell derived hepatocytes for predicting carcinogenicity based on a toxicogenomics gene expression readout has been shown for the first time by Yildirimman *et al.* (2011). The study was performed with the commercially available product hES-HepTM002 (human embryonic progenitor) (Cellestis AB, Göteborg, Sweden, <http://www.cellestis-stemcells.com>). In that study, samples were generated by incubating hES-Hep with fifteen compounds of three different toxicity classes (GTX: genotoxic carcinogens, NGTX: nongenotoxic carcinogens, NC: noncarcinogens) that were chosen from a previously defined body of model compounds causing genotoxicity and carcinogenicity (Vinken *et al.*, 2008). After incubation, treated samples (72-h treatment response at IC10 concentration) as well as control samples were screened with whole-genome Affymetrix Human Genome U133 Plus 2.0 GeneChip arrays, and the experiments were performed by standard methods (Jennen *et al.*, 2010).

An important requirement for all *in vitro* assays is the metabolic competence of the system, in particular the activation of the cytochrome P450 machinery. This is necessary because many chemicals need biotransformation before they can exert their genotoxic or carcinogenic effects. For example, the genotoxic compound benzo-[a]-pyrene (BAP)

Table 1. CYP expression in cellular assay.

Symbol	Control	Genotoxic carcinogens					Non-genotoxic carcinogens					Non-carcinogens				
	hESC	2NF	AFL	BAP	NNK	CYC	MPH	PPX	SPB	TPA	WYE	NFE	DIC	CLO	DMA	TOL
CYP11B2	0.53	0.47	0.26	0.57	0.67	0.49	0.55	0.37	0.39	0.21	0.47	0.58	0.60	0.21	0.70	0.50
CYP17A1	0.46	0.43	0.63	0.35	0.37	0.54	0.58	0.45	0.35	0.69	0.61	0.51	0.43	0.68	0.45	0.55
CYP19A1	0.18	0.37	0.32	0.32	0.36	0.18	0.35	0.23	0.24	0.43	0.08	0.43	0.45	0.01	0.27	0.21
CYP1A1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
CYP2B6	0.05	0.21	0.09	0.10	0.08	0.07	0.10	0.12	0.12	0.09	0.03	0.20	0.10	0.02	0.19	0.14
CYP2C19	0.64	0.75	0.53	0.75	0.76	0.58	0.40	0.73	0.57	0.35	0.22	0.74	0.51	0.52	0.68	0.55
CYP2C8	0.59	0.63	0.80	0.57	0.14	0.46	0.59	0.67	0.77	0.40	0.40	0.74	0.78	0.75	0.75	0.22
CYP2C9	0.09	0.13	0.19	0.20	0.31	0.03	0.64	0.41	0.17	0.37	0.13	0.40	0.16	0.30	0.50	0.22
CYP2E1	0.36	0.47	0.55	0.38	0.42	0.78	0.45	0.40	0.48	0.43	0.64	0.39	0.57	0.25	0.47	0.22
CYP2J2	0.05	0.13	0.05	0.13	0.04	0.24	0.05	0.06	0.24	0.02	0.16	0.12	0.03	0.13	0.04	0.20
CYP3A5	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00

Numbers refer to detection *p*-values from gene expression signals against background noise. Cells in yellow indicate that the gene is expressed/activated with respect to the chemical exposure.

[R. Yildirimman, *et al.* (2011). Reproduced from Oxford University Press.]

needs biotransformation by *CYP1A1* and *CYP1B1* that display similar catalytic activities in converting B[a]P-7,8-dihydrodiol to mutagenic metabolites. Upregulation of these *CYPs* is through binding of BAP to *AHR*, a ligand-activated nuclear transcription factor. Table 1 lists key enzymes for metabolization of chemicals and their expression in the hES–Hep cells with and without chemical exposure.

In total, 8745 genes were found expressed in hES–Hep of which 4148 (47%) were associated with the GO (Gene Ontology) term *metabolic processes* underlining the high level of metabolic competence of the cells.

A general critical question is how similar the gene expression in untreated hES–Hep cells is with respect to the human *in vivo* situation in the liver. The hES–Hep cells were found positive for important hepatocyte markers and liver-related proteins. For example, $\alpha 1$ -AT, AFP, ALB, CK18, and *HNF4 α* were expressed along with *CYP1A2* (Figure 1a). Furthermore, the overall gene expression response correlates with the gene expression in human liver. Pearson correlation was 0.80 and 0.79 as compared to human fetal and adult liver, respectively (Figure 1b). Amongst important phase I–III enzymes, such as *CYP1A2*, *CYP3A4*, *CYP2C9*, and *GST α 1* or *OCT1*, *OATP2*, and *BCEP*, CYP activity measurements confirmed the presence of functional enzymatic activity of the most important hepatic CYP enzymes (*CYP1A2*, *CYP3A4*, *CYP2B6*, *CYP2C9*, and *CYP2C19*). Among the expressed genes, many hepatocyte markers such as *CD44*, *TM4SF1*, *DPP4*, *SERPINA1*/ $\alpha 1$ -AT, ALB, TF, *FOXA2*, *CYP3A5*, *HNF4 α* , *CYP1A1*, AFP, *ABCC2*, *SERPINA7*, *GSTA1*, *KRT19*, *CYP3A4*, and *CYP2B6* were found (Figure 1c).

These results point to the fact that the gene expression in hES–Hep highly resembled human hepatic cell systems.

3 GENE REGULATORY RESPONSES TO CHEMICALLY INDUCED CARCINOGENICITY

To characterize the induced transcriptional response in the stem-cell-based assay at the gene level, a two-step analysis was performed. Firstly, the fold changes for all fifteen chemical treatments were computed in comparison to untreated gene

expression, and a one-way balanced ANOVA (analysis of variance) was conducted in order to identify significantly altered response genes among the toxicity classes as described below:

$$Y_{gij} = \mu_g + GC_i + \varepsilon_{gij}$$

where μ_g is the global expression mean for gene g ; i = GTX, NGTX, or NC refers to the toxicity class; and j to the compounds of the respective class, $j = 1, \dots, 5$. The significance of the variance ratios was quantified with the F-statistic (p -value < 0.05) comparing the between-group variance against the within-group variance. In a second step, Student's t -test for each gene across the set of ANOVA-significant genes was applied to judge whether the gene's fold changes were consistent within the same toxicity class (p -value < 0.05).

In total, 592 ANOVA-significant genes were identified with a significant genotoxic response, in particular comprising genes that act in the interplay of DNA damage response, p53 signaling, and apoptosis. In addition, important tumor suppressors (*PDCD4*, *BCL2*, *SMAD3*, *FHIT*, *ATM*, *TCHP*, *ITGB5*, and *RPL10*) and oncogenes (*RAB17*, *RRAS*, *FAS*, *MDM2*, and *GNA15*) were found. Mechanisms relating to these genes have been shown previously in the literature, for example, the activation of *MDM2* by the downregulation of the tumor suppressor *FHIT* (Schlott *et al.*, 1999). Downregulation of *FHIT* and upregulation of *MDM2* was observable in four of the five genotoxic treatments (AFL, BAP, NNK, and CYC), while such effects were much weaker or not visible with the nongenotoxic and noncarcinogenic treatments. These findings were consistent with recent results from rodent studies, for example, from primary rat hepatocytes (Mathijs *et al.*, 2009), where *Mdm2* has also been prominently identified as discriminating between genotoxic and nongenotoxic compounds.

In order to derive discriminative gene expression patterns, hierarchical clustering of the expression matrix was performed (Figure 2a). Five main clusters of genes were visible (marked as C1–C5). Incorporating promoter sequences of the corresponding genes, for example, using software such as AMADEUS (Linhart *et al.*, 2008), reveals significantly overexpressed transcription factor binding site motifs that can be associated to transcriptional regulatory patterns that direct the gene expression response to chemical carcinogenicity.

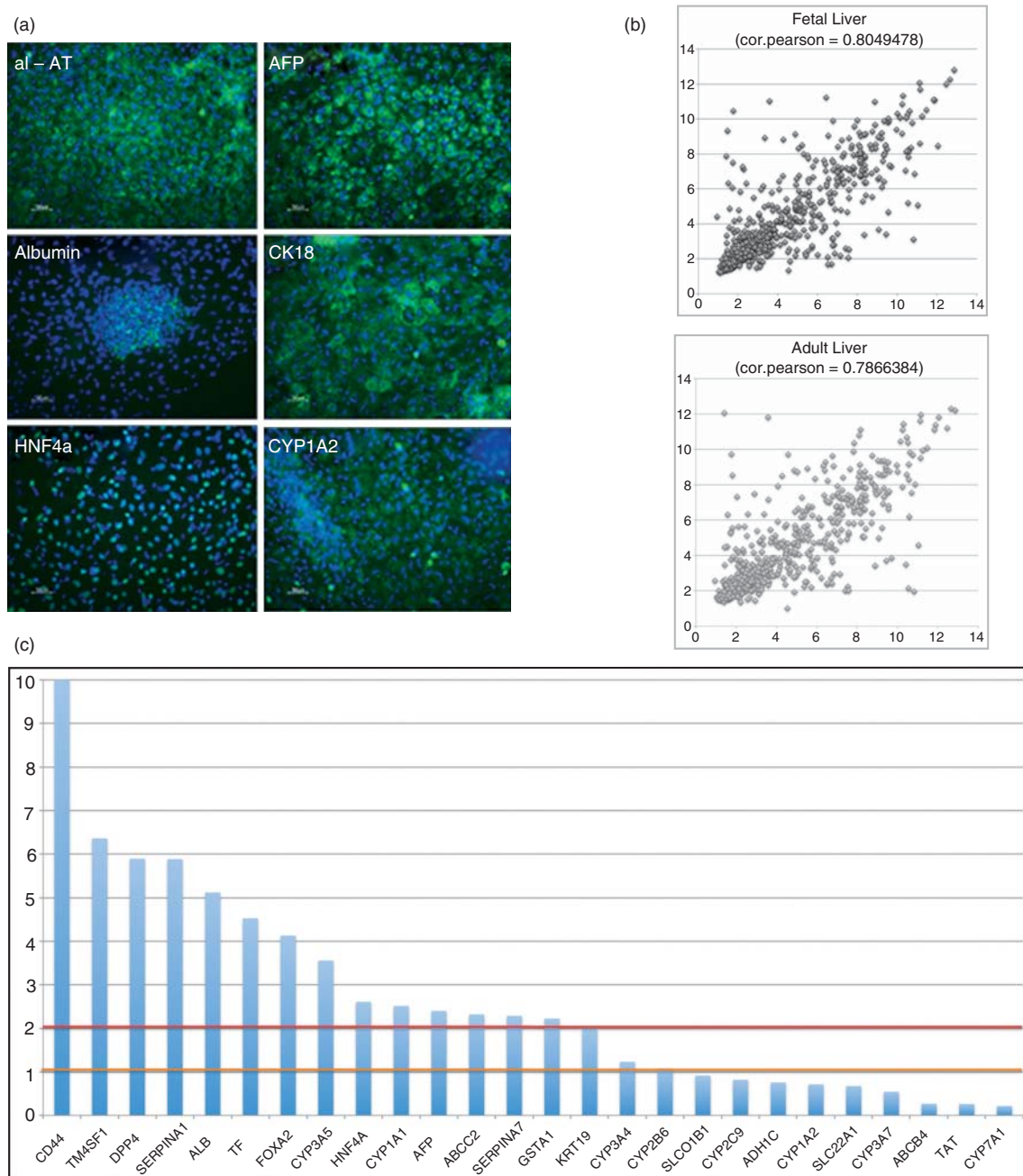


Figure 1. Hepatocyte-like characteristics of hES-Hep. (a) ICC screens showing stem-cell-derived hepatocytes positive for known hepatocyte markers, such as $\alpha 1$ -AT, AFP, Albumin, CK18, HNF4a, and CYP1A2. [R. Yildirimman, *et al.* (2011). Reproduced from Oxford University Press.] (b) Correlation of gene expression of stem-cell-derived hepatocytes with fetal (upper plot) and adult (lower plot) liver; correlation coefficients are 0.80 (fetal liver) and 0.79 (adult liver) based on genes responsive in the chemical treatments. (c) Significance of gene expression of hepatic markers in untreated hES-Hep. Y-axis displays the negative $\log_{10}$ of the detection p -values of the gene expression signals. Horizontal lines indicate the 0.1 (orange) and 0.01 (red) detection levels. [R. Yildirimman, *et al.* (2011). Reproduced from Oxford University Press.]

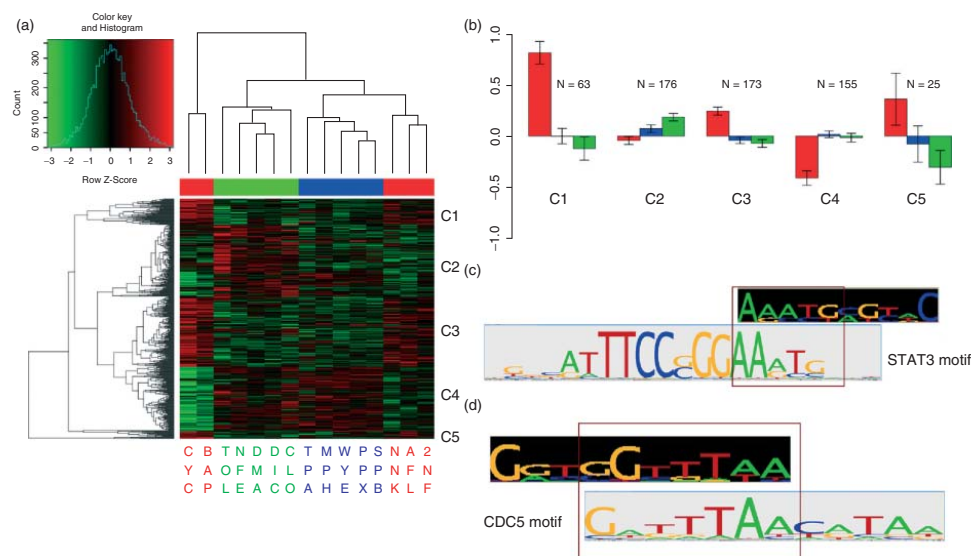


Figure 2. Gene expression and promoter sequence analysis. (a) Hierarchical clustering of response genes (rows) and experiments (columns). Compounds are indicated by colors (red, GTX; blue, NGTX; and green, NC). Clustering was generated with the R/Bioconductor package. [R. Yildirimman, *et al.* (2011). Reproduced from Oxford University Press.] (b) Average profiles among the GTX, NGTX, and NC compounds for each of the five main clusters. Y-axis refers to the average expression ratio (log-2) of all genes related to the respective cluster averaged over all substances of the respective toxicity class. X-axis displays GTX (red), NGTX (blue), and NC (green) profiles for each cluster (C1–C5). Error bars refer to the standard error of the mean. [R. Yildirimman, *et al.* (2011). Reproduced from Oxford University Press.] (c) *De novo* motif identified with a cluster of genes ($N = 63$) upregulated in GTX experiments and downregulated in NGTX and NC experiments and its match to the STAT3 binding motif. (d) *De novo* motif identified with a cluster of genes ($N = 155$) downregulated in GTX experiments and unaffected in NGTX and NC experiments and its match to the CDC5 binding motif. Motifs were identified with the AMADEUS tool (Linhart *et al.*, 2008).

The first cluster (C1, $N=63$) exhibited genes predominantly upregulated with GTX, downregulated with NC, and basically unaffected by NGTX treatments (Figure 2b). A *de novo* motif was found in 32 gene promoters ($p\text{-value} = 1 \times 10^{-14}$), which resembled partly the *STAT3* motif (Figure 2c). Increase of *STAT3* was previously found to contribute to the transformation of a normal hepatic stem cell to a cancer stem cell (Mishra *et al.*, 2009). Genes associated with that motif were found overrepresented in p53- and MAPK signaling pathways. In this cluster, two further motifs were found, which completely matched two binding sites for the transcription factor *HSF* (heat shock factor) involving 42 ($p\text{-value} = 8.4 \times 10^{-16}$) and 41 ($p\text{-value} = 5.2 \times 10^{-15}$) gene promoters, respectively, that were overrepresented in p53- and ATM signaling. This transcription factor induces response to a wide array of harmful agents and is involved in the regulation of numerous genes. Its protective role under carcinogenic environments (AFL and BAP among others) was previously reported (Banerjee *et al.*, 2000). Another cluster (C4, $N=155$) comprised genes predominantly downregulated with GTX and unaffected by other treatments. Genes of this cluster were related to cell cycle pathways and a *de novo* motif was found in 73 gene promoters ($p\text{-value} = 2.9 \times 10^{-15}$) similar to the *CDC5* motif (Figure 2d).

Furthermore, expression of the response genes within each toxicity class was fairly consistent as judged with one-sample Student's *t*-test. In total, 421 of 592 genes were identified as consistently differentially expressed. Furthermore, 301 were selectively altered within a specific toxicity class; of these 153 (51%) were specific for GTX, 60 (20%) for NGTX, and 88 (29%) for NC substances. GTX effects can directly be linked to p53 effectors (i.e., MDM2) and cellular proliferation. Specific GTX genes include TPK1, which has been associated to GTX response (Magkoufopoulou *et al.*, 2012), and is mostly related to cellular growth and DNA damage (Searle *et al.*, 2004). Furthermore, dysregulation of *FAS* was previously related to cellular growth and of *WWOX* related to *TP53* what is in concordance with the study by Scott *et al.* (2011). *WWOX* is an anti-apoptotic gene responsible for tumor growth (Chiang *et al.*, 2013) related to *TP53* and to *TP73* (Lin *et al.*, 2013), which appears downregulated only in the GTX compound class with mean values around zero for NGTX and NC (downregulation

due to GTX exposure was validated by qPCR in Scott *et al.*, 2011).

Given that NGTX are negative for genotoxicity and chronic bioassays are no longer regularly performed, this class of carcinogens is undetected. Therefore, test systems detecting NGTX or their modes of action are required. Of particular interest here are the mechanisms of action which are conserved among different chemicals. Among the genes consistently dysregulated in the NGTX class ($N=60$) were *ATM* and *BCL2*. The plausible role of these genes was suggested in the study by Schaap *et al.* (2012) performed in rat primary hepatocytes. *ATM* is involved in cellular stress responses and DNA damage. *BCL2* is an anti-apoptotic gene that appeared upregulated in the NGTX class enabling cell proliferation. Although the hierarchical clustering does not show a characteristic cluster for NGTX, overrepresented pathways for cluster 4 ($N=155$), formed by genes upregulated in the NC and NGTX classes, include responses that have been previously associated to NGTX mechanisms, that is, dysfunction in the lipid metabolism represented by alterations of the superpathway of geranyl diphosphate biosynthesis I (via mevalonate) and ERK/MAPK targets.

4 IDENTIFICATION OF PREDICTIVE PATHWAYS FOR CARCINOGENICITY

A major goal of systems toxicology is to assemble the functional relationships, or interactions, between toxicants and physical entities in the cell (genes, proteins, metabolites, etc.) and to describe the interaction map of the cellular responses to chemical exposure. Many methods have been developed to measure such interactions and have been applied to human and to animal models. Hundreds of thousands of interactions have been detected, reported in the literature, and assembled in interaction databases; however, these databases are often complementary and tend to focus on one or a few types of interactions, for example, protein-protein or metabolic interactions, while in reality, all the different interaction types coexist in the cell. In order to obtain a global interaction map that reflects biology as completely as possible, subject to the current knowledge, many available interaction resources should be considered. The heterogeneity of databases in terms of interaction type, data model,

and data exchange format makes their integration a daunting task. In order to overcome this heterogeneity, community efforts are ongoing to generate meta-databases that combine the content and the interaction resources in this widespread community.

One of these meta-databases is the ConsensusPathDB (Kamburov *et al.*, 2013; <http://consensuspathdb.org>). In this resource, physical entities (genes, proteins, metabolites, etc.) from different sources are matched depending on their accession numbers and interactions. The web interface of ConsensusPathDB aims to serve as a one-stop shop for searching, visualizing, and retrieving the integrated interaction data, as well as for tools that use these data for interaction- and pathway-centric analysis of genes, proteins, and metabolites (resulting, e.g., from large-scale transcriptomics, proteomics, or metabolomics experiments). The ConsensusPathDB resource integrates the content of 30 different interaction databases and comprises 2144 predefined human pathways.

Genome-wide microarray data can be directly interpreted with these predefined pathways. Methods have been introduced such as overrepresentation analysis employing Fisher's test or gene set enrichment analysis. Yildirimman *et al.* (2011) used the following scoring scheme.

To evaluate a pathway's responses in the stem-cell-based *in vitro* model, a numerical score S_{ij} for each gene i in each treatment j was calculated by applying the equation

$$S_{ij} = |\log_2(\text{fold-change}_{ij})| * |\log_{10}(p\text{-value}_{ij})|$$

The p -values and the fold changes were generated per gene i when comparing the average value of the intensity data of each treatment with the respective controls. These gene scores were further assigned to pathways. The response score for each pathway was defined as the average score derived from all S_{ij} within a certain pathway j . In order to make the pathway response scores comparable across the panel of compound treatment experiments, relative pathway response scores were calculated. This was done by dividing the individual scores for each experiment by the median pathway response score of that experiment.

Pathway analysis allows distinguishing low-responding and high-responding pathway modules for each toxicity class. High pathway responses among the GTX treatments were observed for

p53 and apoptosis pathway modules while NGTX treatments exerted their effects mainly through PI3K/AKT, PPAR, and MAPK signaling. NC treatment responses were less specific.

An ANOVA approach was performed with the pathway response patterns, and it could be shown that the approach was able to achieve a complete separation of the 15 compounds into three distinct groups with a subset of 37 pathways (Figure 3).

In contrast to gene expression patterns that exerted predominantly genotoxic responses in the hES–Hep system, the pathway patterns are more diverse and also specific for nongenotoxic carcinogenicity. An example is the PPAR signaling pathway that was found highly discriminative for the three toxicity classes (ANOVA p -value = 0.0157) and its response was mainly because of the NGTX treatments. PPAR signaling is biologically interesting *per se* due to the fact that (i) it regulates cell proliferation, differentiation, and survival initiating carcinogenesis in different tissues (Michalik, Desvergne, and Wahli, 2004) and (ii) it has a role in lipid peroxidation and activation of ROS, which might lead to secondary effects of genotoxicity (Ellinger-Ziegelbauer *et al.*, 2009).

5 CONCLUSIONS AND OUTLOOK

hES cell technology has high potential for developing *in vitro* hazard assessment assays for carcinogenicity of chemicals as was shown with three representative toxicity classes. Predictive carcinogenic pathway responses based on modules of the apoptosis, MAPK, and p53 signaling pathways have been identified, which build the basis for a mechanistic understanding of chemical carcinogenesis.

The results show consistent pathway regulation in accordance with other previous findings. This is of crucial interest because it opens the possibility to perform the transition of *in vitro* toxicogenomics from a research tool to one used for regulatory risk assessment. However, an *in vitro* method to be accepted as animal alternative for hazard identification has to meet two basic requirements: (i) relevance, that is, the assay needs to provide measures of toxicity which are in sufficiently good accordance with data either reported for humans or obtained in laboratory animals and (ii) reliability, that is, the assay must have a sufficient degree of precision (in the sense of giving consistent results)

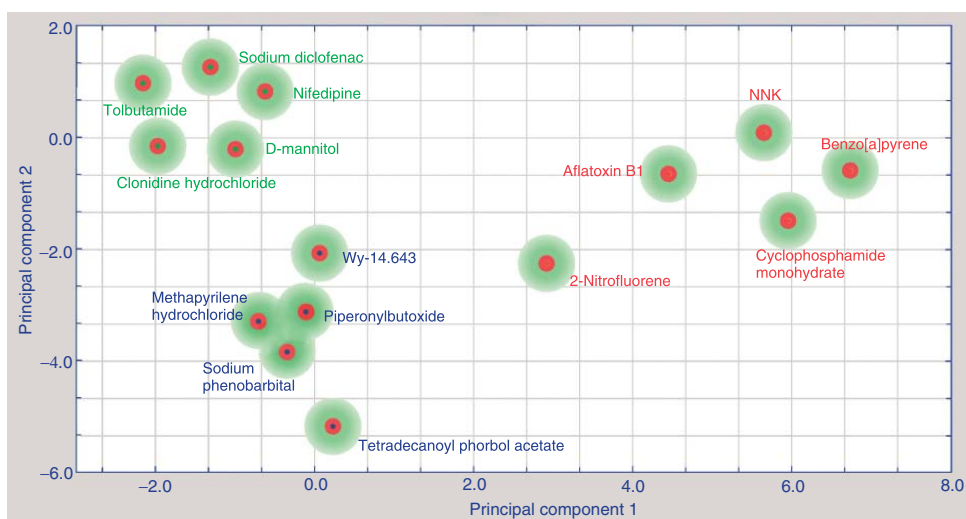


Figure 3. Discrimination potential of carcinogenicity pathways. Principal component analysis (PCA) of different compound classes [red, genotoxic carcinogens (GTX); blue, nongenotoxic carcinogens (NGTX); and green, noncarcinogens (NC)] generated with 37 pathway response patterns for the 15 substance treatments yields a perfect separation of the toxicity classes. Total variance explained is 69.5% (PC1: 49.9%; PC2: 19.6%). [R. Yildirimman, *et al.* (2011). Reproduced from Oxford University Press.]

and be relatively insensitive to minor variations in test procedures, so that results can be repeated independently in different laboratories. In this respect, the hES–Hep model needs further refinement, and additional challenges with more compounds, in order to meet a broader acceptance (Holzhütter *et al.*, 1996).

Nonetheless, promising results also pave the way toward use of stem-cell-derived liver cells for toxicity testing in light of the development of iPS cell technology that will allow new applications in toxicology, which are better suited for testing carcinogenicity in the human system. These human-like cellular systems combined with new -omics technologies such as microarrays or next generation sequencing will revolutionize toxicogenomics. In the future, this will lead to less use of animal experiments and increased possibilities to avoid bringing harmful chemicals to the market.

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Epigenetic Modifications and Stem Cell Toxicology: Searching for the Missing Link

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1 INTRODUCTION

Epigenetics originally meant “on top of genetics.” Initially, epigenetics was considered to play a key role primarily in development and cell differentiation, thus granting cells with identical genomes the ability to develop distinctive phenotypes based on genetic programming (Holliday, 1990). The field has since emerged from an interesting phenomenon to a galvanizing area of research. Today, *epigenetics* is accepted as a term to describe heritable alterations in chromatin structure and gene expression patterns without accompanying changes in DNA sequence. The mechanisms underlying epigenetic chromatin changes include DNA methylation, histone posttranslational modifications (i.e., acetylation, methylation, and ubiquitination), and noncoding RNAs (i.e., microRNA) (Kouzarides, 2007).

In eukaryotic cells, the basic functional unit of chromatin is the nucleosome that consists of a 147 base pair segment of DNA wrapped around a central core of eight histones: two histone H2A–H2B dimers and a histone H3–H4 tetramer. Another type of histone, linker histone H1, binds to DNA between the nucleosomes. Microscopically, the chromatin structure is separated into two regions: (i) heterochromatin, containing tightly packed nucleosomes, facilitated by the heterochromatin protein 1 (HP1); this region typically occurs at centromeres

and telomeres; and (ii) euchromatin, consisting of decondensed areas (Berger, 2007).

The amino-terminal tails of core histones are subject to a variety of posttranslational modifications that influence their interactions with DNA and contribute to the regulation of transcription. Epigenetic histone modifications have best been characterized for histones H3 and H4 and include small covalent modifications such as methylation, acetylation, and phosphorylation. Other modifications include ubiquitination, sumoylation, ADP ribosylation and deimination, and the noncovalent proline isomerization that occurs in histone H3 (Kouzarides, 2007).

Posttranslational histone modifications are dynamic and are modulated by enzymes that aim to either advance or reverse the reactions. Histone deacetylases (HDACs) are a family of enzymes that catalyze the removal of acetyl groups from the ϵ -NH₃ molecules of lysines, and act in conjunction with histone acetyltransferases (HATs). This family of enzymes functions to acetylate lysine groups in nuclear histones (Dawson and Kouzarides, 2012). Histone methyltransferases mediate the addition of methyl groups to arginine (protein arginine methyltransferases, PRMTs) and lysine (histone lysine methyltransferases, HKMTs) residues. Furthermore, the methylation status of arginine is balanced by deiminases, which modify the side chain to citrulline, while histone kinases function to catalyze the phosphorylation of serine and threonine residues.

The best-characterized sites of histone methylation are those that occur on lysine (K) residues. Particular methylation states on the same residue can also localize distinctively. For instance, the mono-methylations of histone 3 (i.e., H3K9m1, H3K27m1, and H3K79m1) are modifications correlated with activation of genes, whereas trimethylations of histone 3 (i.e., H3K9me3, H3K27me3, and H3K79me3) are related to gene repression (Barski *et al.*, 2007).

2 DNA METHYLATION

DNA methylation is a covalent chromatin modification that is marked as the attachment of a methyl group to the 5-position of cytosine (5mC) in a cytosine–phosphate–guanine (CpG) dinucleotide. CpG methylation is symmetrical and is directed toward isolated CpGs, clustered CpGs, or even CpGs within a CpG island. Using vertebrate sequences in GenBank, a CpG island was first proposed as being a 200-bp stretch of DNA with a C + G content of 50% and an observed-to-expected CpG ratio frequency of greater or equal to 0.6 (Gardiner-Garden and Frommer, 1987). Later, employing complete chromosome sequencing, a CpG island was defined as regions of DNA of >500 bp with a G + C content of $\geq 55\%$ and an observed-to-expected CpG ratio of 0.65 (Takai and Jones, 2002). Methylation of the promoter CpG islands is involved in specific events including (i) gene silencing, (ii) genomic imprinting, (iii) X-chromosome inactivation, and (iv) carcinogenesis.

Methylation of mammalian genomic DNA is catalyzed by deoxyribonucleic acid methyltransferases (DNMTs), which play a pivotal role in chromatin remodeling and gene expression regulation. Mammalian DNMTs include DNMT1, DNMT2, DNMT3a, and DNMT3b. The maintenance DNMT1 specifically identifies hemi-methylated DNA following replication and methylates the daughter strand (Jaenisch and Bird, 2003), while DNMT2 activity has not yet been reported to be implicated in DNA methylation but rather is associated with transfer RNA methyltransferase activity. DNMT3a and DNMT3b are correlated with *de novo* DNA methylation that are activated during embryonic development and cell differentiation, and are associated with inactivation of genes whose consequences are no longer needed (e.g.,

genes related to pluripotency) (Turek-Plewa and Jagodzinski, 2005).

Abnormal DNA methylation patterns have been reported for several forms of cancer, and these modifications might be employed as potential biomarkers for tumor categorization. The repression of tumor suppressor genes is directly related to DNA hypermethylation and leads to genomic instability. DNMT inhibitors reverse these effects and provide effective treatment modalities for many tumors. Treatment with DNMT inhibitors such as 5-azacytidine (a nucleoside inhibitor) and decitabine (the deoxyribose analogue of 5-azacytidine) induces cellular DNMT depletion and hinders the action of DNMT such that its inactivation is demonstrated in the format of a covalent protein–DNA adduct (Jones and Taylor, 1980).

3 HISTONE ACETYLATION

Acetylation of lysine residues is a major histone modification involved in transcription, chromatin structure, and DNA repair. The acetylation status of histones is mediated by two classes of two families of enzymes: (i) HDACs are modification enzymes that catalyze the removal of acetyl molecules from ϵ -NH₃ groups of lysines, which balances the action of another family of enzymes, HATs, and (ii) HATs function to acetylate lysine groups in nuclear histones. Thus, a change in the balance of acetylation on chromatin results in alterations in the regulation of gene expression patterns (Marks *et al.*, 2001). While HATs are widely regarded as transcriptional activators because histone acetylation is associated with transcriptionally active chromatin, HDACs are deemed as transcriptional repressors. Human cells contain 18 known HDACs that are divided into four groups: class I (HDAC 1, 2, 3, and 8), class II (IIa, HDAC 4, 5, 7, and 9 and IIb, HDAC 6, and 10), class IV (HDAC 11), and class III (sirtuins 1–7) (Konsoula *et al.*, 2009).

The increased attention toward inhibiting the HDACs as targets for cancer therapy results from their well-established ability to modify several cellular functions that are deregulated in cancer cells. Attenuation of HDACs often leads to cellular differentiation, growth arrest, and apoptosis in a broad spectrum of tumor cells and *in vivo*. A variety of compounds (Konsoula *et al.*, 2011) with HDAC inhibitor activities have been described. Several

histone acetylation and deacetylation markers have also been associated with the progression of some neurodegenerative diseases (Konsoula and Barile, 2012). Structural classifications of these enzymes include (i) short-chain fatty acids (i.e., sodium butyrate, valproic acid), (ii) hydroxamic acids [i.e., suberoylanilidehydroxamic acid (SAHA), H6CAHA, and trichostatin A], (iii) cyclic tetrapeptide (i.e., trapoxin A), (iv) cyclic peptides [i.e., depsipeptide (FR901228) and apicidin], and (v) benzamides (i.e., MS-275). Several of these inhibitors are in phase I and II clinical trials and SAHA (vorinostat) and depsipeptide (romidepsin) have been approved by the US Food and Drug Administration for the treatment of cutaneous T-cell lymphoma (Kelly *et al.*, 2005).

4 STEM CELLS

Stem cells are categorized as follows: (i) embryonic stem (ES) cells, (ii) adult stem cells, and (iii) induced pluripotent stem (iPS) cells. Table 1 summarizes distinctive classes of stem cells.

ES cells are derived from pluripotent cells of the early mammalian embryo and are capable of unlimited, undifferentiated proliferation *in vitro*. In chimeras with intact embryos, ES cells contribute to a wide range of adult tissues including germ cells; pluripotency allows cells to differentiate into many cell types and tissues, incorporating the three embryonic germ layers. Furthermore, experimental evidence has shown that organogenesis of embryonic and adult stem cells to neural, muscle, dermal, and bone marrow lineages is possible. Figure 1 illustrates a general outline of the steps involved in the derivation of differentiated ES cells in culture.

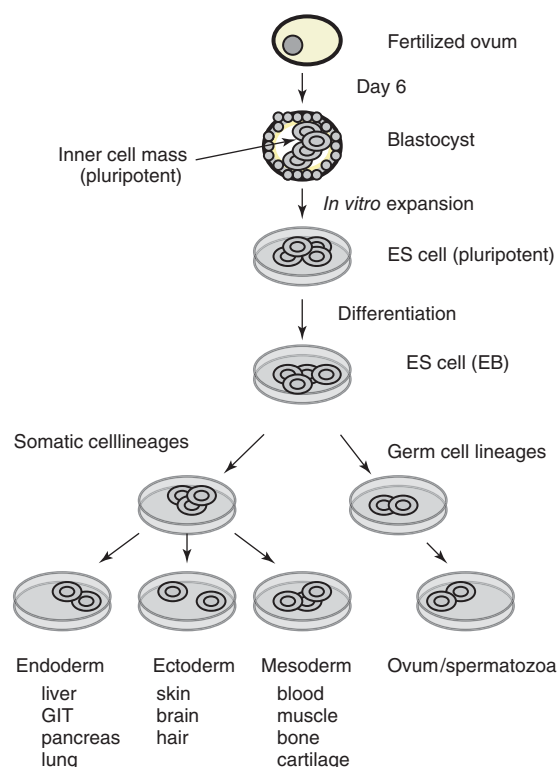


Figure 1. ES cells isolated from the inner cell mass of the blastocyst. Under the influence of different growth factors, ES cells have the potential to differentiate into cells of different lineages (EB, embryoid bodies).

4.1 Culture of Stem Cells

The concept of deriving ES cells from mice and humans is relatively recent. The protocols developed to maintain ES cells in continuous culture continue to emerge. Some progress, however, has demonstrated the transformation of ES cells to different organ and tissue structures including *in*

Table 1. Stem cell types and characteristics.

Stem cell type	Characteristics
Pluripotent cells	Have the capacity to transform into the three germ layers (endoderm, mesoderm, and ectoderm)
Multipotent cells	Possess the ability to differentiate into numerous cell types but within an established lineage (e.g., hematopoietic stem cells)
Embryonic stem cells	Generally accepted as pluripotent cells originating from the inner cell mass of the blastocyst
Adult stem cells	Undifferentiated cells, derived from basal cells within a tissue; exhibit limited self-renewal capability and are multipotent
Cancer stem cells	Small percentage of cells within tumors that demonstrate self-renewal and differentiation potential
Induced pluripotent stem cells	Pluripotent cells generated from differentiated cells by programmed ectopic expression of transcription factors (e.g., Oct4, Sox2, Klf4, and c-Myc)

vitro organogenesis and differentiation of human, primate, and mouse ES cells into functional gut-like organs, smooth muscle cells, cardiomyocytes, neurons, and hematopoietic entities. In addition, soluble factors influence direct differentiation of mouse ES cells. For example, interleukin-3 (IL-3) directs cells toward macrophage, mast cell, neutrophil, or erythroid lineages. Retinoic acid induces neuron formation, and transforming growth factor (TGF) induces myogenesis. Differentiation is also encouraged by culturing ES cells on different feeder layers and extracellular matrix (ECM) components that exhibit morphological and physiological properties characteristic of the target organ.

The increasing awareness of the influence of growth factors (GFs) and basement membrane substrata has improved our understanding of the role of cell regeneration in wound repair and in preneoplastic tumor growth. For instance, in their ability to form tight junction (TJ) strands, differentiating epidermal and epithelial cells act to prevent free exchange of most solutes between luminal and interstitial fluids along the paracellular route (Calabro, Gazarian, and Barile, 2011). Breakdown in the barrier functions of these membranes may contribute to cellular neoplasia. Thus, continuous progress in stem cell biology and biotechnology is based on the understanding that *in vivo* epithelial, epidermal, and mesenchymal cells undergo continuous renewal by multipotent stem cells that remain anchored to the organ basement membrane. Thus, a single stem cell migrates and commits to all of the embryonic lineages.

4.1.1 Influence of Culture Environment on ES Cells

When ES cells are allowed to differentiate in suspension culture or in the absence of feeder layers, they form spherical multicellular aggregates or embryoid bodies (EBs) that contain a variety of cell types. By manipulating combinations of GFs in the presence of ECM components, epithelial- or epidermal-specific gene expression and differentiation are induced.

The mechanism by which GFs and ECM influence cell differentiation or expression of differentiation markers is the subject of much interest. In the presence of GFs in culture, mouse ES cells acquire different expression profiles and display evidence for TJ. It is understood that without GFs, leukemic

inhibitory factor (LIF), or feeder layers, mouse ES cells spontaneously differentiate into a variety of uncontrolled cell colonies, and properties for cellular differentiation are delayed.

Thus, GFs and ECM are capable of directing linear specific differentiation. For instance, epidermal growth factor (EGF) is a mitogenic polypeptide that allows for differentiation into ectoderm (including skin) and mesoderm, while keratinocyte growth factor (KGF) is an epithelial cell-specific mitogen responsible for normal proliferation and differentiation of epithelial cells. Consequently, differentiation of ES cells in culture to specific lineages may be mediated by enrichment of the environment with GFs that influence cell expansion, improved survival, or differentiation.

4.1.2 Culture of Human and Mouse ES Cells

Currently, most experimental stem cells are derived from blastocysts of humans or mice. The cells spontaneously differentiate into embryonic structures in the absence of a feeder layer or conditioned medium. They are maintained in the undifferentiated state by frequent subculture (every 2 days) on confluent mitomycin-C-treated or irradiated mouse embryonic feeder (MEF) layers or in complete culture medium to which is added LIF, or both.¹

In Figure 2, undifferentiated mouse ES cultures are distinguished from differentiated populations and from the underlying MEFs by the formation of EBs. These aggregates are 4–10 cell layers thick over the feeder layer, are oval or round, and have defined borders. As undifferentiated mouse ES cell colonies propagate, they maintain their aggregate morphology (small arrow, Figure 3) and are still distinguished from the feeder layer (asterisk). Figure 4 shows differentiated ES cultures when cells are allowed to come into contact, forming confluent monolayers—a transformation that occurs in the absence of feeder layers and LIF. The figure features the loss of aggregate morphology by ES cells.

Protocols for the preparation of mitomycin-C-treated MEF feeder layers, culture media, serum requirements, and methods for subculturing and maintenance in undifferentiated or differentiated states are described in other toxicology testing texts (Barile, 2013).

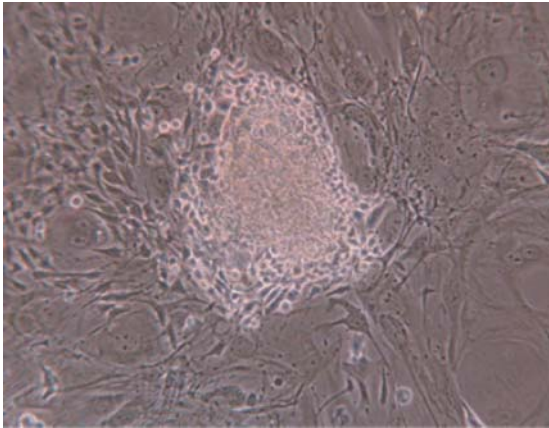


Figure 2. Phase contrast photomicrograph of embryoid bodies (EBs) from mouse embryonic stem cells. Aggregates are round and have well-defined borders. They maintain their clustered morphology and are distinguishable from the underlying feeder layer (400 $\times$).

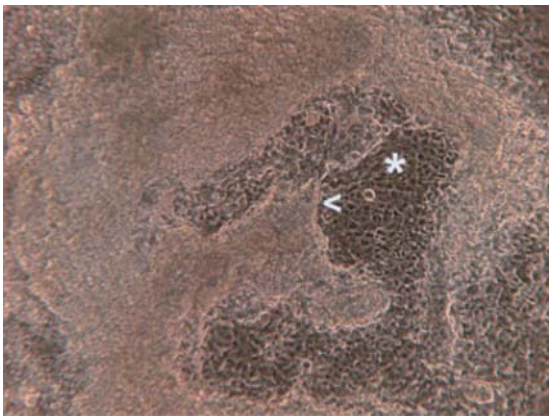


Figure 3. In the absence of feeder layers and LIF, undifferentiated EBs from mouse ES cell colonies propagate. They lose their aggregate morphology (<) and migrate to the periphery (*) as they continue to differentiate (400 $\times$).

4.2 Embryonic Stem Cells

ES cells emerge from the inner cell mass of the blastocyst of an embryo and are characterized by indefinite self-renewal capacity and pluripotent differentiation capability (Evans and Kaufman, 1981). They can be sustained in the undifferentiated state for an extended period of time and maintain normal karyotypes following extended passaging (Keller, 2005). ES cells maintain pluripotency and

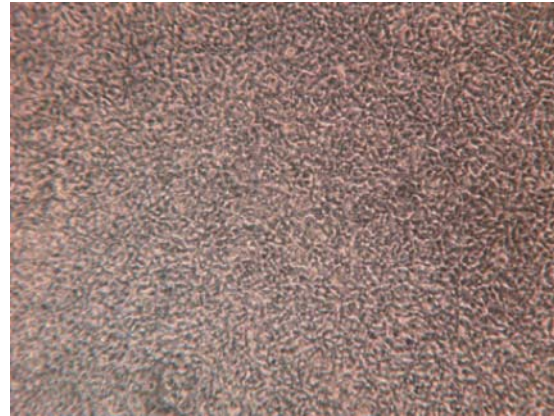


Figure 4. Differentiated mouse ES cells (400 $\times$).

self-renewal status by the interplay of a combination of transcriptional factors including Oct4, Nanog, Sox2, Klf4, Lin-28, and c-Myc. Table 2 provides a short description of transcriptional factors important for pluripotency and self-renewal of stem cells.

Epigenetic posttranslational modifications are known to play key roles in determining the lineage and developmental stage of cells within a multicellular organism (Gadhia, Calabro, and Barile, 2012). ES cells have distinctive epigenetic profiles as compared to differentiated cells (i.e., embryonic carcinoma cells and hematopoietic stem cells). Open chromatin markers, acetylated H3K9, and methylated histone 3 lysine 4 (H3K4) are expressed at higher levels in pluripotent ES cells than in tissue-specific stem cells (Bartova *et al.*, 2008). Other histone modifications, correlated with active chromatin (i.e., H3K14ac, H3K4me3, H3K36me2, and H3K36me3), are also enhanced in ES cells as compared to their derived neuronal progenitor cells (Efroni *et al.*, 2008).

Importantly, several repressive and heterochromatic marks, including H3K9me3, were found to increase during ES cell differentiation (Wen *et al.*, 2009). ES cells carry repressive histone 3 lysine 27 trimethylation (H3K27me3) modifications that prevent the premature expression of lineage-specific genes (i.e., premature expression occurs in embryonic ectoderm development-deficient ES cells) (Azuara *et al.*, 2006). Histone H3K4 (Lys4) is associated with transcriptional activation by recruiting nucleosome remodeling enzymes and histone acetylases (Santos-Rosa *et al.*, 2003), whereas Lys27

Table 2. Summary of transcription factors (TFs) and their descriptions.

TF	Description
Oct-4/POU5f1	Mammalian POU transcription factor (octamer-binding transcription factor 4); presented in blastomeres and ES cells; inactivation of Oct-4 in embryos and ES cells leads to impaired pluripotency and induces differentiation (Nichols <i>et al.</i> , 1998)
Nanog	Present in the morula and inner cell mass of the blastocyst; associated with maintenance of ES cell self-renewal and pluripotency; deficient cells fail to sustain pluripotency and differentiate into extraembryonic endoderm lineage (Mitsui <i>et al.</i> , 2003)
Sox2	Present in the extraembryonic ectoderm and neural stem cells; implicated in self-renewal of ES cells; Sox2 and Oct-4 cooperate with Nanog in human and mouse ES cells self-renewal (Yuan <i>et al.</i> , 1995)
Klf4	Present in multiple tissues; forced induction in ES cells causes restriction of differentiation in erythroid progenitors; can be substituted for other TFs such as Nanog and Lin-28 (Li <i>et al.</i> , 2005)
Lin-28	Conserved RNA-binding protein that is highly expressed in mouse and human embryonic stem cells; used experimentally to augment generation of iPS cells from human fibroblasts (Yu <i>et al.</i> , 2007)
c-Myc	Implicated in stem cell self-renewal and differentiation, specifically in the interaction between stem cells and the local microenvironment; modulates an assortment of other cellular functions, such as cell cycle regulation, differentiation, and metabolism (Murphy, Wilson, and Trumpp, 2005)

methylation is linked to transcriptional repression by endorsing a compact chromatin structure (Ringrose, Ehret, and Paro, 2004). ES cells harbor a novel chromatin modification pattern, termed *bivalent domains*, that contains large regions of Lys27 methylation and smaller regions of Lys4 methylation (Bernstein *et al.*, 2006).

In undifferentiated human ES cells, the DNA is nonmethylated, whereas as cells differentiate, they undergo rapid DNA methylation (Lagarkova *et al.*, 2006). Concomitantly, Oct4 and Nanog promoters shift from histone 3 lysine 4 trimethylation (H3K4me3), which are linked to transcriptional activation, to H3K27me3 marks, which are associated with repression (Pan *et al.*, 2007). Conversely, Oct4 and Nanog demonstrate coordination and cooperation with HDAC1/2 and Metastasis Associated 1/2, in a network termed *NODE* (*Nanog and Oct4-associated deacetylase*). This association encompasses HDAC activity comparable to NuRD (nucleosome remodeling and deacetylase) complex (Liang *et al.*, 2008).

4.3 Adult Stem Cells

Adult stem cells emerge from a multitude of adult tissues (e.g., bone marrow, blood vessels, skin, muscle, and bone). They retain multipotent differentiation ability, characteristic of the tissues of lineage. For instance, neural stem cells exist in the subventricular zone and hippocampus regions (Conrad and Huss, 2005), whereas hematopoietic

and mesenchymal stem cells are abundant in the bone marrow (Pittenger *et al.*, 1999).

Cancer stem cells (CSCs) are described as a distinctive subset of cells that maintain stem-like properties and encompass the potential to trigger tumor heterogeneity when transplanted in an experimental model. Furthermore, by employing an assortment of markers, CSCs are derived from an extensive array of human cancers and are distinguished accordingly. Table 3 summarizes CSC tumor stem cell population markers for leukemia (Bonnet and Dick, 1997), breast (Al-Hajj *et al.*, 2003), brain (Singh *et al.*, 2004), myeloma (Matsui *et al.*, 2004), prostate (Collins *et al.*, 2005), lung (Kim *et al.*, 2005), pancreatic (Li *et al.*, 2007), colon (O'Brien *et al.*, 2007; Ricci-Vitiani *et al.*, 2007), head and neck (Prince *et al.*, 2007), and ovarian (Baba *et al.*, 2009) cancers.

By comparing DNA methylation and chromatin structures between CSCs and differentiated breast and liver cancer cells, the DNA methylation status and repressive histone modifications (i.e., H3K27me3) were shown to be markedly lower in CSCs as compared to their “differentiated” counterparts (Yasuda *et al.*, 2010). For example, CD133 transcription is modulated by histone modifications and inversely correlates with DNA methylation at the ovarian tissue active P2 promoter. Treatment of CD133⁺ ovarian cancer cells with decitabine, a DNMT inhibitor, or trichostatin A, a HDAC inhibitor, augmented the cell surface CD133 expression (Baba *et al.*, 2009).

Table 3. Cancer stem cell (CSC) markers.

Cancer	CSC markers	References
Leukemia	CD34 ⁺ /CD38 ⁻	Bonnet and Dick (1997)
Breast	CD44 ⁺ /CD24 ^{-low} /lineage	Al-Hajj <i>et al.</i> (2003)
Brain	CD133 ⁺	Singh <i>et al.</i> (2004)
Myeloma	CD138 ⁻	Matsui <i>et al.</i> (2004)
Prostate	CD44 ⁺ /α ₂ β ₁ ^{hi} /CD133 ⁺	Collins <i>et al.</i> (2005)
Lung	Sca-1 ⁺ /CD45 ⁻ /pecam ⁻	Kim <i>et al.</i> (2005)
Pancreatic	CD44 ⁺ /CD24 ⁺ /ESA ⁺	Li <i>et al.</i> (2007)
Colon	CD133 ⁺	O'Brien <i>et al.</i> (2007) Ricci-Vitiani <i>et al.</i> (2007)
Head and neck	CD44 ⁺	Prince <i>et al.</i> (2007)
Ovarian	CD133 ⁺	Baba <i>et al.</i> (2009)

These findings are indicative of the ability of epigenetic modification of CD133 and its correlation with the induction of pluripotency-related genes in mouse neurosphere following combined treatment with decitabine and trichostatin A (Ruau *et al.*, 2008).

The demethylating agent, 5-azacytidine, has been shown to strengthen the overall repopulating potential of cultured umbilical cord blood CD34⁺ cells. This phenomenon is attributed to demethylation of relevant silenced genes (Suzuki *et al.*, 2004). HDAC inhibitors (e.g., valproic acid and trichostatin A) increase both proliferation and self-renewal of normal hematopoietic stem cells, thus allowing for the potential use in the treatment of acute myeloid leukemia (Bug *et al.*, 2005).

It is also interesting to note that CSCs contrast with normal adult stem cells in that they do not preserve the “bivalent chromatin motif” (Yasuda *et al.*, 2010). These bivalent domains tend to coincide with transcription factor (TF) genes that are expressed at low levels. Thus, they appear to be important in silencing developmental genes in ES cells while keeping them poised for activation. The lack of these regulatory regions in CSCs implies the loss of developmental regulation in the differentiation process. Additionally, studies investigating the DNA methylation facets of CSCs indicate that the epigenetic markers of CSCs parallel the ES cells paradigm rather than the adult normal stem cell pattern (Wong *et al.*, 2008).

Delineating the epigenetic reprogramming events that regulate CSC properties and discerning the variations between CSCs and normal adult stem cells will facilitate identification of novel drugs that selectively target CSCs without affecting normal tissue homeostasis.

4.4 Induced Pluripotent Stem Cells

iPS cells are actualized by genetic reprogramming of differentiated somatic cells (i.e., fibroblasts, neural cells, and liver cells) into dedifferentiated ES cells, by employing ectopic expression of an amalgam of transcriptional factors (Takahashi *et al.*, 2007). Regulating the transcriptional expression network is pivotal as a means to maintain self-renewal and pluripotency of ES cells and as a method to display the full therapeutic potential of these cells. Reprogramming of fibroblasts to pluripotent stem cells can be stimulated *in vitro* by the ectopic expression of the following set of TFs: (i) Oct4, Sox2, c-Myc, and Klf4 (Takahashi *et al.*, 2007); (ii) Oct4, Sox2, Nanog, and Lin-28 (Yu *et al.*, 2007); and (iii) Oct4, Nanog, Sox2, Lin-28, c-Myc, and Klf4 (Liao *et al.*, 2008).

Somatic cells reprogrammed to iPS cells reposition posttranslational histone modifications to an ES cell-like status. For example, HDAC inhibitors, such as trichostatin A and valproic acid, employed to trigger active histone acetylation marks, augment iPS cells reprogramming efficiency (Huangfu *et al.*, 2008). Furthermore, to sustain pluripotency, the promoters of genes encoding Nanog, Oct4, and Sox2 show a high degree of H3K4 methylation, leading to a transcriptionally active chromatin state (Pan *et al.*, 2007).

During the reprogramming process, somatic cells also reset their pattern of DNA methylation to an ES cell-like phase—DNA of transcriptionally active genes (i.e., pluripotency genes) is hypomethylated, while silenced genes are hypermethylated (Meissner *et al.*, 2008). Inability to demethylate pluripotency genes correlates with intermediate or partial states of reprogramming, and knock-down of the maintenance methyltransferase DNMT1 or treatment

with 5-azacytidine, a DNA methylation inhibitor, converts intermediate states to full pluripotency (Kim *et al.*, 2010).

5 STEM CELLS IN TOXICOLOGY TESTING

Pluripotent stem cells have found utility in drug and chemical toxicity screening, particularly to assess the effect of compounds with regards to potential toxicity on diverse human cell lines (Dholakiya and Barile, 2013). This development entails the induction of human pluripotent stem cells into cells of specific tissue that are selectively targeted by the investigative compounds, followed by evaluation of dose–response toxicity assays. Currently, the principle of therapeutic lineage reprogramming (i.e., trans-differentiation) has been investigated in various arenas of regenerative medicine. Table 4 presents examples of *in vitro* reprogramming using the lineage-reprogramming approach.

Evidence to date shows that the lineage-reprogramming approach could be applied for some specific cell lineages, such as pancreatic β -cells generation. For instance, it has been demonstrated *in vivo* that pancreatic exocrine cells can be stimulated to metamorphose to pancreatic endocrine β -cells following transfection with an adenovirus that expresses genes for three TFs: Pdx1 (pancreatic and duodenal homeobox factor-1), Ngn3 (Neurogenin3), and MafA (v-maf musculoaponeurotic fibrosarcoma oncogene family, protein A) (Zhou *et al.*, 2008). This intervention involving insulin producing β -cells by direct differentiation may pioneer a potential intervention for treating type 1 diabetes.

Recent findings provide additional proof-of-principle that cardiomyocytes can be generated from human somatic cells by TF-modulated fate instructions. For instance, mouse fibroblast cells differentiated into cardiac cells (Ieda *et al.*, 2010) following transformation with an amalgam of TFs: Gata4 (binds to the DNA sequence “GATA”), Tbx5

(T-box TF), and Mef2c (myocyte-specific enhancer factor 2C also known as *MADS box transcription enhancer factor 2*). This trans-differentiation of fibroblast cells into heart cells has vital implications in directing the field of heart developmental biology and might provide a promising therapeutic pathway in human cardiovascular diseases.

Similarly, mouse fibroblasts can be reprogrammed into functional neuronal cells *in vitro* following transfection with lentivirus expressing a transcriptional network of genes: Ascl1 (achaete-scute homolog 1), Brn2 [POU (Pit-Oct-Unc) domain-containing transcription], and Myt1l (myelin TF 1-like) (Vierbuchen *et al.*, 2010). This procedure has the capability to reprogram cells toward neuronal properties, such as intrinsic excitability and execution of action potentials.

Formation of induced dopaminergic neurons, a cell type compromised in Parkinson’s disease, from somatic cells might have significant implications for unraveling critical processes for neuronal development and cell replacement treatment. Induced dopaminergic neurons are derived from mouse and human fibroblasts by two sets of protagonists: (i) employing a combination of TFs (Ascl1, Brn2, and Myt1l) and neural conversion factors [Lmx1a (LIM homeobox TF 1 alpha) and FoxA2 (forkhead box A2)], (Pfisterer *et al.*, 2011); and (ii) applying three TFs: Mash1 (mammalian achaete-scute homolog 1), Nurr1 (nuclear receptor-related 1 protein), and Lmx1a (LIM homeobox TF 1, alpha) (Caiazzo *et al.*, 2011). The induced neurons manifest electrophysiological traits in accordance with midbrain dopaminergic neurons.

6 CONCLUSIONS

The principles of epigenetics comprise a network of mechanisms including histone modifications, DNA methylation, chromatin remodeling, and noncoding RNAs. Epigenetic approaches have

Table 4. Examples of *in vitro* cell reprogramming and associated transcription factors (TFs).

Differentiated somatic cells	TF	Induced pluripotent cells	References
Pancreatic exocrine cells	<i>Pdx1, Ngn3, MafA</i>	Pancreatic β cells	Zhou <i>et al.</i> (2008)
Fibroblasts	<i>Gata4, Tbx5, Mef2c</i>	Cardiomyocytes	Ieda <i>et al.</i> (2010)
Fibroblasts	<i>Ascl1, Brn2, Myt1l</i>	Neurons	Vierbuchen <i>et al.</i> (2010)
Fibroblasts	<i>Mash1, Nurr1, Lmx1a</i>	Dopaminergic neurons	Caiazzo <i>et al.</i> (2011)
Fibroblasts	<i>Ascl1, Brn2, Myt1l, Lmx1a, FoxA2</i>	Dopaminergic neurons	Pfisterer <i>et al.</i> (2011)

contributed a wealth of scientific knowledge on mechanisms regulating gene expression with respect to development, cell differentiation, and pathology. Furthermore, numerous studies are currently elucidating the epigenetic landscape underlying ES and somatic stem cells. Hopefully, other aspects of stem cell function and the molecular frame of pluripotency will be able to be investigated from the discoveries generated from the epigenetic studies. The elucidation of *in vitro* as well as *in vivo* epigenetic mechanisms will have important ramifications for further advances in the stem cell research field and will eventually lead to novel targets for therapeutic interventions.

END NOTES

1. Recombinant human LIF is a lymphoid factor that promotes long-term maintenance of mouse ES cells by suppressing spontaneous differentiation; it is not effective in human ES cells.

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Epigenetic Modeling and Stem Cells in Toxicology Testing

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1 INTRODUCTION

Epigenetics serves as an integral link between genetics and developmental biology, which until recently were considered to be independent (Holliday, 2006). Epigenetics is often referred to as the *study of heritable modifications* in gene expression without altering the DNA sequence. Historically, the field was responsible for the understanding of cell differentiation and was seen as a fundamental link between phenotypic characteristics of the cells and their genotype. As early as 1975, the heritability of alterations in gene function was examined in studies on yeast, fungi, and mammalian cell cultures. These revelations clearly demonstrated that the trans-generational inheritance of genes occurs through mitosis of somatic cells (Holliday, 2006). In addition, epigenetics has served to explain features of disease states that, to date, have not been adequately understood, including its importance in the progression of cancers, neurodegenerative diseases (Konsoula and Barile, 2012), and cardiovascular disorders and diabetes (Jiang, Bressler, and Beaudet, 2004). Furthermore, environmental factors and behavioral habits of the diet and nutrition affect epigenetic states to initiate various changes.

The Dutch Hunger Winter study (1976) demonstrated that maternal malnutrition suffered by the Dutch population in 1944 resulted in subsequent

effects on adult health, thus providing some crucial support for the fetal basis of adult disease (FeBAD) theory (see the following sections). In addition, dietary alterations occurring during embryogenesis alter methylation patterns of various genes, thus varying their expression (Schulz, 2010). The theory hypothesizes that repercussions of nutritional and non-nutritional perturbations occurring during the early stages of development, at times, are seen only in the latter stages of life and may extend beyond the first generation of offspring. Consequently, the *in utero* environment establishes the basis for adult development, influences metabolism and hormonal homeostasis, and prepares the organism for subsequent cell expansion and proliferation (Calkins and Devaskar, 2011).

Numerous epidemiological studies have been performed on animals and humans in order to investigate the effects of various insults *in utero*. In addition, *in vitro* studies are also important for understanding genetic and epigenetic mechanisms. The use of embryonic stem (ES) cells obtained from the inner cell mass (ICM) of the blastocyst is one of the best approaches to study embryotoxicity and epigenetic changes, owing to their pluripotency and self-renewal properties (Calabro, Konsoula, and Barile, 2008). Furthermore, germ line stem cells also serve as an efficient model for studying epigenetic changes occurring during development (Perera and Herbstman, 2011).

2 EPIGENETICS

2.1 Introduction

Unlike genetics, which deals with changes in gene sequence and inheritance, epigenetics is associated with the mechanisms of inheritance without affecting DNA sequences, which may or may not be reversible (Kovalchuk and Kovalchuk, 2012). In 1942, Conrad Waddington coined the term *epigenetics* and presented it as a critical link between genotype and phenotype (Waddington, 1942; Jablonka and Lamb, 2002). Because *Waddingtonian* epigenetics was considered a hypothetical model of how phenotypic transformations occur following genetic alterations in organisms during the intricate process of development, the field was mysteriously misinterpreted as a developmental biology phenomenon (Roloff and Nuber, 2005). Today, it is common knowledge that epigenetics addresses situations where genetic modifications and phenotypic differences do not necessarily correlate. In fact, *Waddingtonian* epigenetics would fit appropriately within traditional cell biology, at the junction of genetics, developmental biology, and cell biology. Consequently, the terminology has undergone numerous revisions (Jablonka and Lamb, 2002; Roloff and Nuber, 2005). Interestingly, the commonality between the traditional ideas of

historic *Waddingtonian* epigenetics and its current interpretation is the focus on alternative developmental pathways, including the intricate and detailed processes, the underlying stability yet flexibility of the pathways, and the interplay of environmental factors affecting the cell (Jablonka and Lamb, 2002). Furthermore, Waddington's epigenetics dealt with the mechanism of phenotypic characters arising from the genotype of the organism during the process of development (Waddington, 1957; Bird, 2007). Conversely, and most importantly, Riggs and colleagues defined epigenetics as the study of heritable changes in gene function, induced through the processes of mitosis or meiosis, without corresponding alterations in DNA sequences (Russo and Riggs, 1996; Bird, 2007; Goldberg, Allis, and Bernstein, 2007; Cortez and Jones, 2008; Berger *et al.*, 2009; LeBaron *et al.*, 2010).

2.2 Chromatin Structure

The study of chromatin and its structure is an important aspect of epigenetics because of its influence on biological function and transcriptional regulation (Cortez and Jones, 2008, Figure 1). As the changes are heritable, it is equally important to understand the mechanisms of chromosomal initiation, maintenance, and heritability. This accounts for the thrust

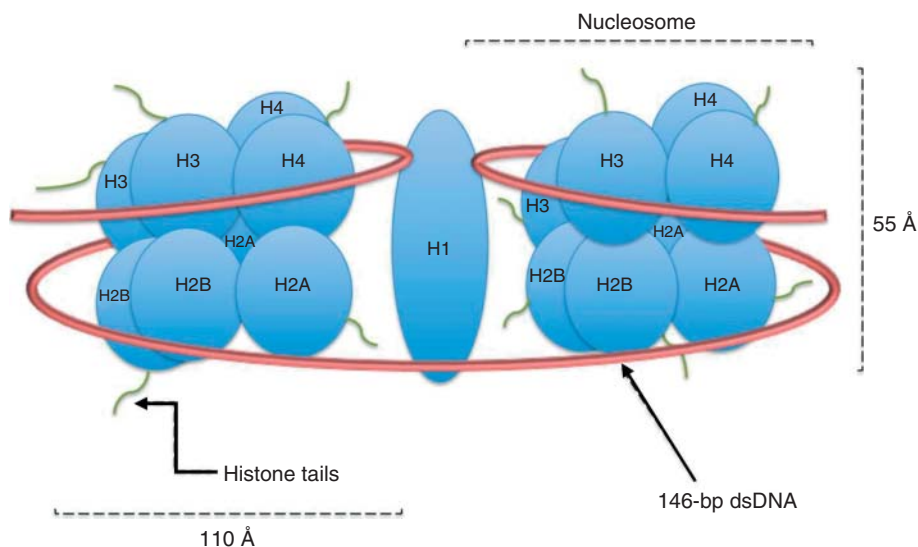


Figure 1. Structure of chromatin. The 146-bp dsDNA is wrapped around the histone octamer, 1.8 turns, to form the nucleosome—the fundamental repeating unit of chromatin.

of recent interest in the field, generating a plethora of information about chromatin and its functional characteristics (Berger *et al.*, 2009).

Chromatin is the complex of DNA and associated proteins, together with histones and nonhistone proteins, which comprise the epigenetic landscape of the cell (Goldberg, Allis, and Bernstein, 2007; Cortez and Jones, 2008). Chromatin exists in two different functional states: (i) heterochromatin, which is the condensed and inaccessible, transcriptionally inactive and repressed, silent state (Heitz, 1929; Roloff and Nuber, 2005), and (ii) euchromatin, which is the decondensed, transcriptionally active state, principally because the DNA is open and accessible (Weintraub and Groudine, 1976; Roloff and Nuber, 2005). Regulation of gene expression (i.e., regulated transcription) entails chromatin remodeling, DNA methylation, histone modifications, and replacement of core histone proteins by variant histones, nonhistone protein

modifications, and noncoding RNA molecules (Goldberg, Allis, and Bernstein, 2007; Collas, 2009). The two main mechanisms involved in regulating gene expression at the chromatin level include (i) DNA methylation, occurring at cytosine residues, and (ii) posttranslational modifications (PTMs) of histones, which occur at amino terminals of the histone proteins (Cortez and Jones, 2008; Arita and Costa, 2009; Collas, 2009). Furthermore, maintenance of the epigenetic state in multicellular organisms is important for proper development and formation of the embryo (Issa, 2007).

Epigenetic modifications induce changes in gene expression and transcription either autonomously or in conjunction with other modification mechanisms (Berger, 2007; Ho and Tang, 2007; Gadhia, Calabro, and Barile, 2012) (Table 1). Two possible pathways by which modifications affect the process of transcription are (i) by affecting the chromatin state (i.e., formation of heterochromatin or euchromatin),

Table 1. Epigenetic modifications and effects on gene transcription and associated diseases.

Epigenetic modification	Alteration	Effect on transcription	Genes affected	Disease implicated
DNA methylation	CpG island hypermethylation	Repression	MLH1	Colon, endometrium, and stomach cancers
			BRCA1	Breast cancer
			MGMT	Several tumors
			NEP	AD ^a
			DR3	RA ^b
	CpG island hypomethylation	Activation	MASPIN S100P	Pancreatic cancer
Histone modifications	CpG island shore hypomethylation	Repression	SNCG	Breast and ovarian cancer
			MAGE	Melanoma
	H3 and H4 deacetylation	Repression	PAD12	MS ^c
			HOXA2 GATA2	Colon cancer
	H3K4me3 demethylation	Repression	CDKN1A	Tumor formation
	H4K20me3 demethylation	Loss of heterochromatic structure	HOX genes	Various cancers
	H3K9me3 and H3K27me3	Repression	Sat2, D4Z4	Various cancers
	Aberrant acetylation	Diverse	CDKN2A, RASSF1	Various cancers
	Aberrant methylation	Diverse		PD ^d , HD ^e
	Aberrant phosphorylation	Diverse		HD ^e , FA ^f , DT1 ^g
				AD ^a

^aAlzheimer's disease.

^bRheumatoid arthritis.

^cMultiple sclerosis.

^dParkinson's disease.

^eHuntington's disease.

^fFriedrich's ataxia.

^gDiabetes type I.

which in turn affects binding of transcription factors to DNA polymers, and (ii) by affecting the organization of chromatin-binding proteins (Berger, 2007). It is widely accepted that these modifications are heritable, that is, transferred through several generations; their onset is as early as the beginning of fetal development and it is quite possible that their effects are not seen until adulthood (Ho and Tang, 2007).

2.3 DNA Methylation

DNA methylation refers to the covalent addition of methyl functional groups at one of the nucleotide bases of the DNA sequence via *S*-adenosylmethionine (SAM). The process is catalyzed by one or more deoxyribonucleic acid methyltransferases (DNMTs), following DNA replication. Additionally, in mammals, methylation of cytosine at position 5 is very well characterized (Issa, 2007; Li, Guo, and De, 2012). DNA methylation is also known to affect cellular global hypomethylation or hypermethylation at promoter sites, leading to gene silencing and loss of protein function (Issa, 2007). Cytosine residues undergoing methylation are located in the CpG dinucleotide, which cluster together to form CpG islands. These complexes occupy about 500 bases in length—3 kb in size and located abundantly across the genome. They appear at about every 100 kb and, more importantly, are present in nearly half of human genes. The importance of DNA methylation is evident by its ability to regulate gene expression as well as influencing embryogenesis and tumorigenesis. Methylation states vary with different tissues, with various differentiation states, disease state and its progression, and temporal stage. Approximately, 50% of CpG islands are located in the promoter region of the “housekeeping” genes, which necessarily are unmethylated to maintain the active state of the genes. Thus, methylation state of the islands will only sequentially affect transcription. Interestingly, both hypomethylated and hypermethylated states organize the conditions necessary to promote a disease state (Li, Guo, and De, 2012).

DNMTs are the enzymes responsible for covalent modification. Four types of DNMTs are capable of influencing the degree of methylation: DNMT1, DNMT3A, DNMT3B, and DNMT3L, each accountable to a specific function in methylation. Briefly, DNMT1 is responsible for maintenance

of methylation; DNMT3A and DNMT3B are required for *de novo* methylation while DNMT3L has a regulatory role, in cooperation with other methyltransferases, as it does not have any enzymatic activity of its own (Li, Guo, and De, 2012). In addition, DNMT3L is responsible for activation of DNMT3A and DNMT3B *in vivo* (Gopalakrishnan, Emburgh, and Robertson, 2008).

Because DNMTs are associated with regulation of methylation states, it is plausible that their activity or expression influences the degree of methylation and associated disease states. For example, removal of either DNMT1 or DNMT3 decreases genome-wide methylation by 3 and 4% only, respectively. However, removal of both demethylase enzymes decreases methylation by 98%. Like most other enzymatic reactions, methylation patterns are influenced by temperature, as it is one of the important physical properties for optimum reactivity. Disease states, such as *Helicobacter pylori* infections, alter DNA methylation conditions leading to gastric cancer, while smoking increases the risk of hypermethylation (Li, Guo, and De, 2012). In addition, DNMT1 is the most abundant methyltransferase recognized in cancer cells. Continuous expression of methyltransferases is crucial, principally because inhibiting expression results in serious consequences; that is, complete loss of methylation states in newly dividing cells leads to re-expression of suppressed genes (Issa, 2007). For instance, mice lacking the enzyme isoform *Dnmt3A* die within four weeks of their birth, while those lacking *Dnmt3B* die during the embryonic stage due in part to concomitant developmental defects. Additionally, mice devoid of *Dnmt3L* isoform also show major developmental deficiencies, thus confirming that the methylation situation is important for proper embryogenesis and development in mammals (Gopalakrishnan, Emburgh, and Robertson, 2008).

In addition to DNMT overexpression, aberrant hypermethylation of tumor-specific genes at the promoter regions affects cell cycle, cell differentiation, cell adhesion, DNA repair, and apoptosis. These expression profiles are of fundamental utility as markers for various disease states. For example, abnormal DNA methylation affects chromosomal stability, DNA repair (e.g., mismatch), and hypomethylation and hypermethylation of oncogenes and tumor suppressor genes. As it is

understood that oncogenes and tumor suppressor genes are significant regulators of the development of cancer, and methylation states predispose normal cells to suppress gene expression, it is reasonable to conclude that this regulatory pathway is a hallmark of cancer. Alternatively, the degree of gene methylation is capable of determining whether it acts as a tumor suppressor gene or an oncogene (Li, Guo, and De, 2012). However, it is important to distinguish that in association with pathologic states, DNA methylation affects many genes, thus making it a nonspecific mechanism of cellular transformation and toxicity (Issa, 2007).

Various drugs that act as DNA methylation inhibitors include (i) nucleoside inhibitors, (ii) non-nucleoside inhibitors, and (iii) rationally designed inhibitors. Nucleoside inhibitors comprise cytosine analogues such as 5-azacytidine and 5-aza-2'-deoxycytidine, both of which are capable of degrading DNMTs. However, for these compounds to be effective, the cells should either be in the DNA synthesis phase of the cell cycle or require incorporation of DNA. Essentially, the cells must be actively dividing. Non-nucleoside inhibitors are older therapeutic compounds, such as hydralazine and procainamide; the mechanism by which they inhibit methylation is not yet clear. Their use, however, is limited as these drugs are not capable of inhibiting hypermethylation to a large extent. Although the information available for the third category of inhibitors is relatively new, it appears that they require the activity of more than one gene for DNMT inhibition. Because there is significant chatter amongst DNMT pathways, it is reasonable to conclude that targeting only one enzyme does not result in complete inhibition of methylation (Issa, 2007).

As a therapeutic target, DNA methylation profiling is a nonspecific mechanism (Issa, 2007; Li, Guo, and De, 2012). Colorimetric and fluorimetric analysis, methylation sensitive restriction endonuclease treatment, and polymerized chain reaction (PCR) are analytical techniques used to study genome-wide methylation and individual CpG methylation—two different approaches for studying methylation profiling (Li, Guo, and De, 2012).

2.4 Histone Modifications

Histone proteins regulate cellular processes including gene expression, transcription, DNA

repair, replication, recombination, and segregation (Ramakrishnan, 1994; Lennartsson and Ekwall, 2009). Figure 1 illustrates the arrangement of these alkaline proteins in an octomeric configuration, as they appear in eukaryotic organisms, thus forming the fundamental unit of the chromosome (Smith, 1991; Siebel, Fernandez, and El-Osta, 2010). Their abundance is variable and so is their variety (Sneekes *et al.*, 2009). The histone octomer complex consists of dimers of H3 and H4, flanked by H2A and H2B, respectively. They are present in the core of the nucleosome, the building block of chromatin. H1 acts as a linker histone protein for the octamer, cylindrical in shape with a diameter of 70 Å and thickness of 50–55 Å. Additionally, a 146-bp DNA strand wraps around the octamer, which occupies a significant portion of the cell nucleus (Smith, 1991; Shi, 2007). Of the various types of histone proteins, none are similar in amino acid sequence (Shi, 2007). However, unlike the large differences in the five types of histone proteins, subtypes are at variance in just one amino acid, except H4, which does not have any subtype. Hence, there is a striking similarity amongst subtypes in physical properties and mass. The main biological function of histone proteins, therefore, is the formation of scaffolds for binding to chromatin remodeling proteins in order to compact the DNA, resulting in considerable influence on transcription.

Core histones undergo various covalent modifications at the N-terminal tails, such as acetylation, methylation, phosphorylation, ubiquitination, biotinylation, SUMOylation (small ubiquitin-like modifier), and ADP (adenosine diphosphate) ribosylation (Berger, 2007; Shi, 2007; Suganuma and Workman, 2008; Munshi *et al.*, 2009). These PTMs may occur simultaneously and are capable of affecting one or multiple tails, resulting in a “histone code” that triggers specific downstream pathways along with unambiguous biological effects (Table 2) (Lennartsson and Ekwall, 2009). In addition to the PTMs mentioned earlier, other histone protein variations involve assorted amino acids that undergo modifications. The variations are influenced by the degree of modification and result in a multiplicity of combinations—usually stable, occasionally transient. For example, methylation occurs at either arginine (R) or lysine (K) residues, while methylation occurring at the latter is in the mono-, di-, or tri-methylated state. Similarly, arginine assumes mono- or di-methylated states (Shi,

Table 2. Histone modifications and effects on transcription.

Histone modifications	Histone position	Effects on transcription
Methylation	H3K4	Activation
	H3K9	Repression or activation
	H3K27	Activation
		(mono-methylation)
		Repression (di- and tri-methylation)
	H3K79	Activation
	H3R2	Activation
	H3R17	
	H3R26	
	H4R3	
Phosphorylation	H4K20	Silencing
	H3S10	Activation
Ubiquitination	H2BK120	Activation
	H2BK123	
	H2AK119	Repression
Acetylation	H3K56	Activation
	H4K16	

2007; Lennartsson and Ekwall, 2009). Moreover, acetylation and methylation transpire on arginine and lysine residues and phosphorylation occurs on serine (S) and threonine (T) residues. Alternatively, SUMOylation and ubiquitination occur on lysine residues only along with biotinylation (Munshi *et al.*, 2009). Thus, the structure and function of chromatin is altered either by PTMs of histone or by replacement of core histone proteins by variant histones (Collas, 2009).

2.4.1 Histone Acetylation

Histone acetylation is one of the most studied histone modifications. It is associated with nucleosome conformation as well as transcription regulation. Allfrey, Faulkner, and Mirsky (1964) and Pogo, Allfrey, and Mirsky (1966) were first to propose the effects of histone acetylation on transcription in eukaryotic cells. They described the process of how acetylation of lysine and/or arginine residues on the histone tails of core histone proteins neutralizes their net positive charge, thereby decreasing the affinity of core histone for double-stranded DNA. As a result of this nucleosomal event, there is increased transcriptional activity (Lee *et al.*, 1993; Vettese-Dadey *et al.*, 1996; Struhl, 1998). Acetylation is a covalent modification catalyzed by histone acetyl transferases (HATs), which occur on both arginine and lysine residues. HATs are

divided into two classes: (i) type A HATs acetylate all histone proteins and successively regulate chromatin structure as well as gene expression and (ii) type B HATs acetylate cytoplasmic histone proteins, especially H4K5 and H4K12 (Munshi *et al.*, 2009). The effects of HATs on chromatin structure are reversed by histone deacetylases (HDACs), resulting in a repressive phenomenon (Khochbin *et al.*, 2001). Additionally, HATs are known to function as *co-activators of transcription*, thereby regulating transcription. However, localized histone acetylation of promoter and enhancer regions has also been reported (Eberharther and Becker, 2002).

In vivo, altered chromatin structure is known to affect DNA synthesis and DNA repair, whereas inhibition of HDACs affects cellular processes such as cell proliferation, apoptosis, differentiation, and DNA synthesis. Overall, histone acetylation is associated with transcriptional activation, whereas deacetylation is considered transcriptionally repressive. However, there exists substrate specificity amongst the acetylases and deacetylases affecting the histone tails (Struhl, 1998). In addition to site-specific histone acetylation, global histone acetylation is also necessary for transcriptional regulation; however, both are not sufficient to make chromatin completely accessible, thus requiring other chromatin remodeling enzymes. Interestingly, repressive histone deacetylation is often followed by methylation of the corresponding residue, which further renders chromatin inaccessible for transcription (Eberharther and Becker, 2002).

In addition to effects on transcription regulation, histone acetylation is also fundamental in establishment and maintenance of the differentiated phenotype, as well as supporting chromosomal integrity during the cell cycle and cell transformation (Wade, Pruss, and Wolffe, 1997). Furthermore, the functional importance of HATs and HDACs is evident in developmental processes, deregulation of which results in various human diseases and is linked to progression of several cancers (Eberharther and Becker, 2002).

Various techniques are useful in studying qualitative and quantitative changes in histone modifications. Typically, they include specialized gel systems, incorporation of radioactive precursors, Edman microsequencing, antibody-based detection, and mass spectrophotometry. Edman microsequencing is used for monitoring initial modifications, while antibody-based detection and mass

spectrophotometry are more precise techniques for identification of transformations on histone tails (Munshi *et al.*, 2009).

2.4.2 Histone Methylation

Histone methyltransferases (HMTs) catalyze covalent modification of histones, predominantly occurring at H3 and H4 and affecting lysine and arginine residues. As previously described, lysine residues are mono-, di-, or tri-methylated while arginine residues are mono- or di-methylated substituents (Berger, 2007; Suganuma and Workman, 2008). Moreover, H4 undergoes symmetric as well as asymmetric methylation patterns (Berger, 2007). The most common methylation sites on the lysine residues are H3K4, H3K9, H3K27, H3K36, H3K79, and H4K20, each of which are capable of inducing different biological effects (Berger, 2007; Suganuma and Workman, 2008). Histone modifications and their corresponding biological effects, such as transcriptional activation or repression, along with DNA damage, have since been shown to be coordinated. Until recently, before the identification of various histone demethylases acting on targets such as H3K4, H3K9, H3K27, and H3K46, methylation marks on histones were considered irreversible. For example, the trimethyl state of H3K27 (H3K27me₃) is one of the most stable histone modifications, but discovery of a demethylase for this modification suggests that even the most stable modification is dynamic in nature (Suganuma and Workman, 2008).

Methylation of lysine residues is catalyzed by histone lysine methyltransferases (HKMTs), containing a SET [su(var), enhancer of zeste, trithorax] domain of approximately 130 amino acids, which transfers a methyl group from S-adenosyl methionine (SAM), a cofactor, on to the specific lysine residues, thus resulting in the formation of mono-, di-, and tri-methyl states (Lee *et al.*, 2005; Gucione *et al.*, 2007; Kirmizis *et al.*, 2007; Völkel and Angrand, 2007). The HKMTs are divided into several *families*: (i) SET1, (ii) SUV39, (iii) SET2, (iv) EZH, (v) SMYD, (vi) PRDM, (vii) other SET domain-containing proteins, and (viii) non-SET HMT families (Völkel and Angrand, 2007). Interestingly, histone lysine tails undergo a characteristic burst in mono-methylation levels initially, followed by a slower conversion to di- and/or tri-methyl states, suggesting that mono-methylation is a prerequisite

for latter modifications (Scharf, Barth, and Imhof, 2009). Methylation states (mono-, di-, and tri-) of H3K27 are catalyzed by EZH2—a catalytic subunit of the polycomb repressive complex 2 (PRC2) containing the SET domain and acting as an active methyltransferase site (Rea *et al.*, 2000; Simon and Lange, 2008). Additionally, the trimethyl states of histones are favorable for subsequent binding of structural proteins, such as HP1 and Polycomb, which are capable of inducing chromatin condensation. As trimethylation occurs slowly, chromatin condensation is also brought about gradually, thus preventing premature condensation of chromatin. This deliberate process ensures a stable shift in gene expression, as is often observed in stem cells. Furthermore, trimethyl states assist in predicting the age of cells in culture, as cells that are unable to divide attain senescence and reportedly have higher levels of trimethyl states. In addition, histone methyl modification patterns force changes within the cell cycle (Scharf, Barth, and Imhof, 2009). Thus, in conjunction with other PTMs, histone lysine modifications alter chromatin structure and affect functional outcomes, including transcription and translation (Gadhia, Calabro, and Barile, 2012).

2.4.3 Histone Phosphorylation

The tails of core histones are susceptible to phosphorylation and dephosphorylation, which are mediated by kinases and phosphatases, respectively, at serine (S), threonine (T), and tyrosine (Y) residues. Additionally, the processes are an integral part of the “histone code” and affect several downstream targets. Histone phosphorylation is important for DNA repair, transcription regulation, chromatin compaction, and epidermal growth factor (EGF) signaling pathways. Unlike mammals, H2AX variant histone is absent in yeast, hence H2A is phosphorylated instead at serine 139. This modification is important for various DNA damage response pathways, reportedly nonhomologous end joining, homologous recombination, and replication-coupled DNA repair, processes which are observed throughout the cell cycle. However, removal of H2AXS139 modification from the chromatin, at the end of DNA repair, allows for efficient recovery of cells from DNA damage-induced cell cycle arrest. Similarly, phosphatases for H2AXS139 are also required for DNA repair and recovery. In addition, H2AX variant histone is apparently phosphorylated at tyrosine

142, the modification present throughout the cell, which decreases post DNA damage. Thus, DNA repair that is mediated via H2AXS139 requires dephosphorylation at H2AXY142. Furthermore, phosphorylation of H2AXS139 (H2AXS139ph) is followed by H4S1 phosphorylation, around DNA double-strand breaks, suggesting that the latter is involved in DNA damage response toward the later stages. H4S1 phosphorylation results in deacetylation of H4, which is usually seen at the end of DNA repair (Rossetto, Avvakumov, and Côté, 2012).

Phosphorylation of histone residues is known to affect transcription thereby altering gene expression, often proliferative genes. Phosphorylation of H3S10, H3S28, and H2BS32 is linked with EGF-mediated gene transcription and expression of a few proto-oncogenes (except H3S28). In mice, phosphorylation of H3T6 and H3T11 is related to androgen-mediated transcription regulation and DNA damage. As phosphorylation of H3S10, H3T11, and H3S28 affects H3 acetylation, they are responsible for transcription activation. Moreover, H3S28 phosphorylation is important for H3K27 acetylation in activating transcription, particularly by removal of PRC complexes, resulting in demethylation of H3K27. Furthermore, H3T11 and H3T36 phosphorylation results in demethylation of H3K9 via JMJD2C. These examples demonstrate that H3 tails undergo phosphorylation, which in turn affects *cis* acetylation and methylation patterns, resulting in altered gene expression. Recently, overlapping communication pathways involving phosphorylation and other modifications has been shown to be responsible for repressed transcription and cell cycle arrest as a result of DNA damage. Linker histone H1, also undergoes phosphorylation at various residues, triggering altered chromatin stability and interference with transcription rates. Apparently, H1 phosphorylation is involved in ribosomal biogenesis and cell growth regulation (Zheng *et al.*, 2010; Rossetto, Avvakumov, and Côté, 2012).

Originally, H3 phosphorylation was associated with chromosome compaction during mitosis and meiosis. However, the process has more recently been associated with formation of euchromatin and heterochromatin. Of the marks on H3, H3S10 is the most studied, probably because of its association with mitosis and meiosis in eukaryotes. Heterochromatin proteins (HP) HP1 α , HP1 β , and HP1 γ , bound to methylated H3K9, the latter of which is primarily found in silenced regions, dissociate from H3K9

upon phosphorylation of H3S10 during the M phase of the cell cycle. Additionally, phosphorylation of H1 is reportedly higher in M and S phases of the cell cycle and is associated with chromatin elongation. More recently, histone phosphorylation is correlated with caspase-3 activation and DNA fragmentation, suggesting a role in apoptosis (Rossetto, Avvakumov, and Côté, 2012).

2.4.4 Histone Ubiquitination

As the least understood epigenetic modification, histone ubiquitination involves covalent binding of ubiquitin (Ub), a 76 amino acid protein, to the histone tails of chromatin (Goldknopf *et al.*, 1975; Zhang, 2003). The information transmitted via this molecule depends on the degree of ubiquitination; hence, it serves as a signaling molecule. Many processes, such as cell cycle regulation, protein degradation, and transcriptional regulation, have been linked to ubiquitination. Ubiquitin binds to a lysine residue with the help of various enzymes responsible for activation (E1), conjugation (E2), and ligation (E3). Until the discovery of E2 and E3 proteins, polyubiquitination was considered nonexistent. Among the studies that have reported extensive cross talk amongst various histone modifications, histone ubiquitination has been consistently associated with histone methylation. Functional analysis of histone modification pathways established that both ubiquitination and deubiquitination are transcriptionally activating modifications, with their effects reconciled by histone methylation (Zhang, 2003).

Approximately 5–15% of total H2A is ubiquitinated, in contrast to 1–2% of H2B; however, the latter is widely distributed throughout eukaryotic organisms. Even though H2A is polyubiquitinated, the majority is found as monoubiquitinated, which most commonly occurs at lysine 119 (K119). Unlike H2A, H2B is only found in the monoubiquitinated state, whereas H1 and H3 ubiquitination sites (rare events) have yet to be determined. Like methylation and acetylation, ubiquitination of histones is also a reversible process. However, ubiquitination of histone proteins involves sequential action of E1, E2, and E3 enzymes. Interestingly, E1 enzymes are nonspecific in their actions toward proteins, whereas E2 and E3 are protein specific. Alternatively, deubiquitination is mediated by a group of enzymes known as *isopeptidases* or *deubiquitinating enzymes*

(DUBs), consisting of ubiquitin C-terminal hydrolases and ubiquitin-specific processing proteases. Furthermore, there is a positive correlation between histone ubiquitination and transcription activation. For instance, ubiquitinated H2A and H2B (uH2A and uH2B) are located around the first exon of the actively transcribed dihydrofolate reductase (DHFR) gene in mice and transcriptionally active hsp70 genes, containing up to 50% uH2A. In addition, uH2A and uH2B are also located near transcriptionally active sequences in bovine thymus, chick embryo, and *Tetrahymena* macronuclei. Remarkably, histone ubiquitination affects transcription by three mechanisms: (i) by affecting chromatin folding, (ii) by functioning as a signaling molecule, and (iii) by affecting other histone modifications. Although it is a small molecule, upon entering the nucleosome, ubiquitin is presumed to affect chromatin stability in addition to influencing cell cycle progression (Wu, Kohn, and Bonner, 1981; Zhang, 2003).

3 STEM CELLS

3.1 Introduction

The development of *in vitro* methods to culture plant and animal cells began in earnest only within the last 50 years, the goal of which was to establish living cells and tissues outside of viable organisms. It was not until the 1960s that cell biologists successfully maintained cultured cells outside an intact organ, and only after excessive refinement of techniques. Most of the initial problems centered on safeguarding cells against microbiological contamination, ensuring that the addition of unknown growth factors (GFs) could support continuous cell proliferation, controlling optimum ambient conditions for cell survival (Barile, 2013).

During the early 1980s, pluripotent cells from mice embryos were isolated, paving the path for culturing of ES cells. Stem cells from mouse, as well as from human and nonhuman primates, were obtained from the ICM of the preimplantation embryo at the blastocyst stage (Friel, Sar, and Mee, 2005). The cells were instrumental in studying gene function analysis following the induction of targeted genetic modifications (Solter, 2006).

Stem cells possess properties of pluripotency and self-renewal without aging; that is, the former is

defined as the ability to differentiate into different germ layers and subsequent cell types (Friel, Sar, and Mee, 2005). *In vivo*, these properties are physiologically important for tissue repair following trauma or senescence or for cell turnover in rapidly proliferating tissue, for example, bone marrow and epithelial layers. In addition, stem cells eventually decide between self-renewal and pluripotency with the influence of a highly regulated microenvironment and signaling pathways. The degree of self-renewal varies with the organ type. For example, neuronal stem cells do not undergo extensive self-renewal, compared to most other rapidly replicating cells, such as epithelial or blood cells.

3.2 Stem Cell Biology

Stem cells are an ideal model to understand embryonic development and tissue regeneration. Mouse embryonic stem (mES) cells are cultured as a continuous cell line. Human embryonic stem (hES) cells have also shown immense promise in regenerative medicine. During the course of embryonic growth, cells begin the differentiation process, from the formation of the zygote to development of various organs. The sequence of events is summarized accordingly—the fertilized ovum forms a morula followed by the blastocyst stage at about day 6 post-fertilization. The blastocyst contains the inner cell mass (ICM), from which the ES cells are obtained. Under the influence of various GFs and extra cellular matrices (ECMs), ES cells differentiate into primary germ layers and primordial germ line cells (Wobus and Boheler, 2005, Figure 2, Table 3). *In vitro* ES cells form embryoid bodies (EBs) in suspension cultures or multicellular aggregates (MAs) in monolayer (adhesion) cultures (Calabro, Konsoula, and Barile, 2008, Figure 3).

Increasing awareness of the influence of GFs and basement membrane substrata has improved our understanding of the role of cell regeneration in wound repair and in preneoplastic tumor growth. In their ability to form tight junction strands, differentiating epidermal and epithelial cells act to prevent free exchange of most solutes between luminal and interstitial fluids along the paracellular route. A breakdown in the barrier functions of these membranes contributes to cellular neoplasia. Thus, continuous progress in stem cell biology and biotechnology is based on the understanding that *in*

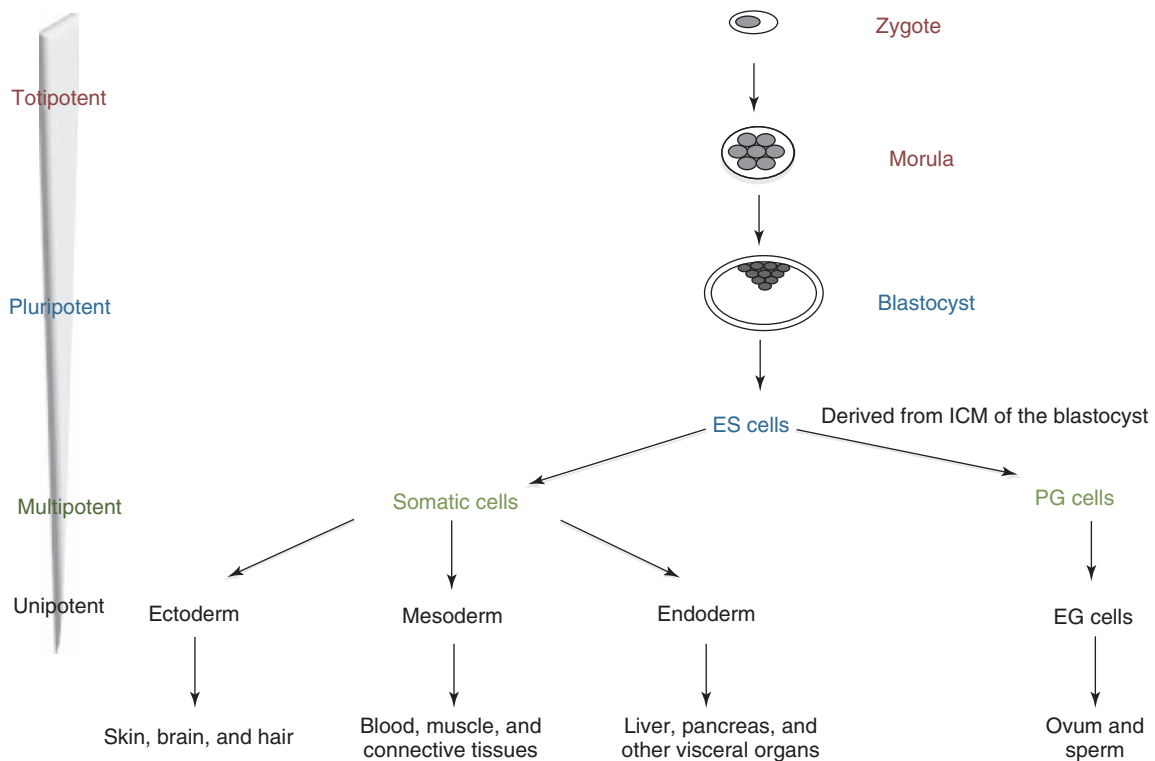


Figure 2. Diagram of derivation and differentiation of ES cells. Under the influence of various growth factors (GFs) and extracellular matrices (ECMs), ES cells differentiate into primary germ layers and primordial germ line cells. The vertical triangular marker on left indicates the decreasing scale associated with differentiation as cells continue to proliferate and approach terminal status.

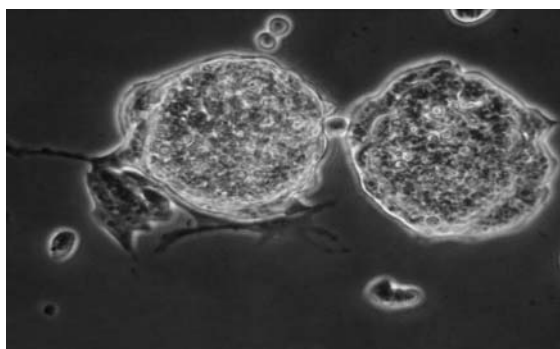
vivo epithelial, epidermal, and mesenchymal cells undergo continuous renewal by multipotent stem cells that remain anchored to the organ basement membrane. It is not surprising therefore that a single stem cell migrates and commits to all of the embryonic lineages.

In vitro cultivation of mES cells involves isolation and culture of preimplantation blastocysts onto feeder layers of mitotically inactivated mouse embryonic fibroblasts (MEFs) in the presence of a GF, leukemia inhibitory factor (LIF), responsible for self-renewal (Figures 4 and 5). Additionally, mES cells have a characteristically stable and normal karyotype, short G₁ phase of the cell cycle, and low population doubling times. Across the scientific community, many groups have used monolayers of inactivated MEF cells or LIF, independently, while some have used both, to ensure that mES cells remain undifferentiated in culture. In addition, LIF has little effect on growth rates but has an extensive role in self-renewal. Chemically, LIF is a cytokine

of the interleukin-6 (IL-6) family, which exerts its actions via the STAT3 signaling pathway. ES cells pluripotency is regulated by many transcription factors and cell surface markers, expression of which is critical *in vivo* as well as *in vitro*. Differential expression of a variety of transcription factors determines the fate of the stem cells; up to twofold upregulation of Oct-4/POU5f1 (POU domain transcription factor for undifferentiated cells and trophectoderm layers) results in potential differentiation of ES cells to mesoderm and endoderm layers, whereas cells lose pluripotency with simultaneous differentiation into trophectoderm because of the loss of Oct-4 expression. Furthermore, Nanog and Oct-4 act as differentiation markers and ES cell identity markers. *In vivo* mES cells form teratomas—that is, tight, rounded, multilayer clusters which *in vitro* aids in the formation of cystic EBs. In addition, MEK/ERK, Wnt, and JAK/STAT are signaling pathways that have a pivotal role in self-renewal and differentiation of ES cells. Hence, disruption of any

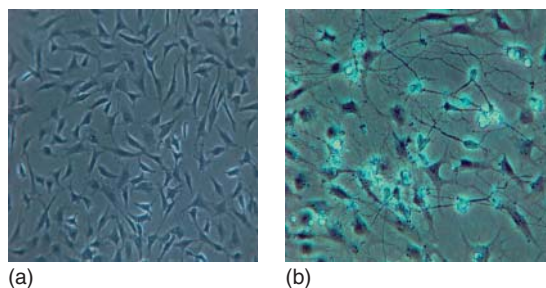
Table 3. Summary table associated with Figure 2.

Terms	Definitions
Teratoma	Tumor consisting of different types of cells, but not belonging to original tissue
Embryonic stem (ES) cells	Pluripotent cells derived from inner cell mass of the preimplantation blastocyst; form teratomas when transplanted into animals
Embryonic carcinoma (EC) cells	Pluripotent cells derived from undifferentiated germ line tumor cells
Embryonic germ line (EG) cells	Derived from gonadal ridge of the embryo or fetus older than 8 weeks of development; do not form teratomas when transplanted into animals
Totipotent	Cells that differentiate into all cell types including extraembryonal and placental
Pluripotent	Cells possessing the ability to differentiate into all three germ layers
Multipotent	Cells possessing the ability to differentiate into more than one cell type but with limited capacity (primordial cells)
Unipotent	Cells with the potential to differentiate into only one type of tissue (terminal differentiation)

**Figure 3.** Phase contrast photomicrograph of embryoid bodies (EBs) from mouse embryonic stem cells. ES cells isolated from the inner cell mass of the blastocyst have the potential to differentiate into cells of different lineages (magnification 400 $\times$) (Gadhia, Calabro, Barile, 2012).

of these factors results in loss of ES cell identity. In addition, while other markers are expressed in ES cells, not all are exclusive to ES cells, and their role in pluripotency as well as self-renewal has yet to be determined (Wobus and Boheler, 2005).

hES cells were successfully isolated and cultured in early 1990s, nearly a decade after isolation of mES cells. Both mES and hES cells share a few fundamental properties, including Oct-4 expression, telomerase activity, extensive proliferation, and formation of teratomas *in vivo* in nude mice. hES cells express cell surface markers SSEA-3 and SSEA-4, whereas mES cells express SSEA-1. Unlike mES cells, hES cells have a longer population doubling time and are unresponsive to the influence of LIF in its ability to inhibit differentiation *in vitro*. Interestingly, however, hES cells activate the LIF/STAT3 pathway, which contributes

**Figure 4.** Phase contrast photomicrograph of differentiated mES cells. After incubation with a variety of GFs, mES cells are directed into primitive germ layer lineages and eventually into specialized cells, such as neuronal progenitor cells (a), which mimic unique developmental traits expressed *in vivo*. Further application and treatment with GFs allows continued progression of primordial cells toward terminally differentiated neurons (b) or astrocytes (magnification 400 $\times$) (unpublished observations).

to relatively higher expression of LIF signaling pathway cofactors and relatively lower expression of differentiation markers (Oct-3/4 and Sox-2) than mES cells. Additionally, in culture, hES cells form flat, loose cellular aggregates along with cystic EBs that express a heterogeneous variety of cell markers.

Self-renewal is an important property of stem cells in order to ensure the survival of higher organisms, as tissues undergo insult, damage, and aging continuously. Hence, cells respond by duplicating all of its contents using a two-step sequential process of cell division, which involves mitosis (nuclear division), followed by cytokinesis (cytoplasmic division). The *cell cycle* ensues and consists of four successive phases, where cells spend most of the time in interphase (G_1 , S, and G_2 phases) (Alberts *et al.*,

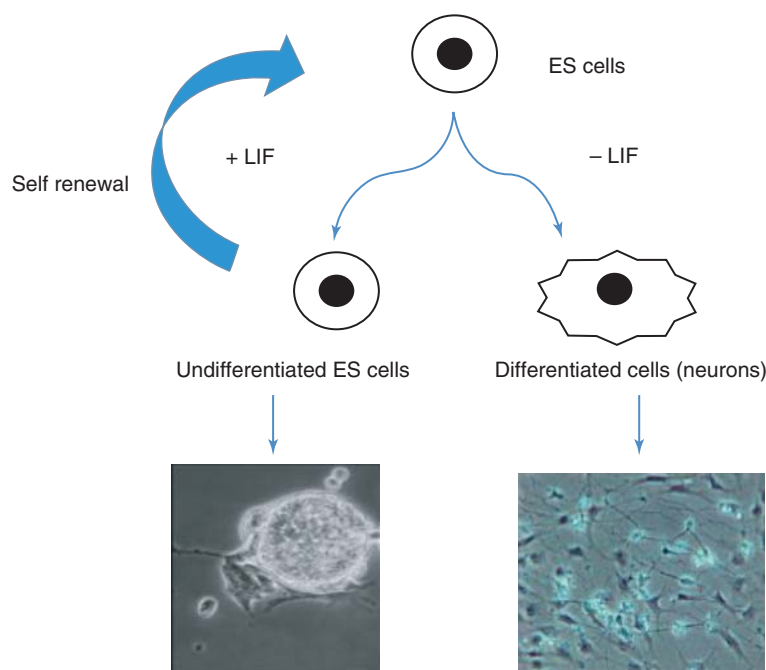


Figure 5. Differentiation fate of mES cells in the presence and absence of LIF.

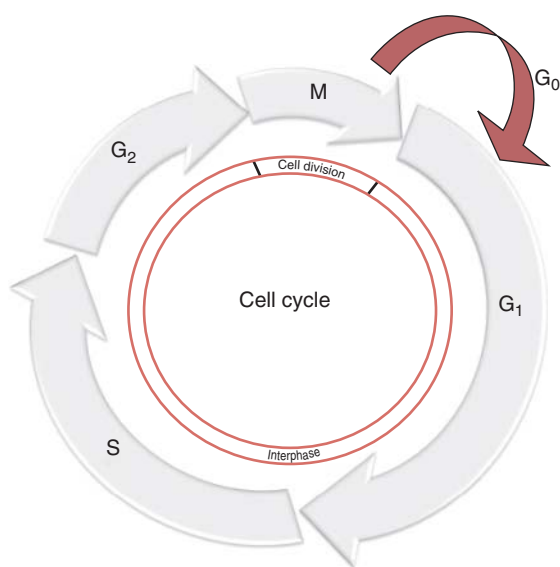


Figure 6. Cell cycle progression of cells *in vitro* and *in vivo*.

1983). Before dividing, cells undergo a preparatory phase, known as *gap phase* (G_1), usually lasting for several hours. The amount of time that cells reside in G_1 predominantly determines the length

of the cell cycle. Following G_1 , cells undergo DNA synthesis (S phase), followed by the completion of chromosomal replication during G_2 . Finally, cells enter mitosis (M phase), where cytokinesis marks the completion of the cell cycle (Barile, 2010, 2013, Figure 6). Interestingly, unlike the translational pathway associated with most structural and secretory proteins, S phase also includes synthesis of histone proteins, along with DNA synthesis. It is important to note that the amount of histone protein produced is approximately equal to the amount of DNA synthesized in the formation of a chromatin fiber, which eventually forms the nucleosome (Alberts *et al.*, 1983). In addition to higher metabolic activity, rapidly proliferating ES cells have a short G_1 phase. Hence, the majority of the cells (60–70%) are normally found in S phase (Savatier *et al.*, 2001).

3.3 Stem Cell Applications in Toxicology

The ES cell serves as an ideal model for studying embryogenesis, embryotoxicity, and developmental toxicities, as they can be either genetically manipulated or allowed to spontaneously differentiate into

various cell types. Mutations resulting from gene targeting help in elucidating gene functions. Modified culture conditions such as monitoring GFs, ECMs, LIF, serum concentrations, and chemicals are tools that determine differentiation status of the cells. In addition, the cells are directed into primitive germ layers and eventually into specialized cells, such as neuronal progenitor cells, which mimic unique developmental traits *in vivo* (Figure 4). *In vitro*, formation of EBs or MAs marks the onset of differentiation of mES cells, preparing for further differentiation into more common cell types, such as cardiac, smooth, or skeletal muscle cells.

Several GFs, particularly IL-3 (Wiles and Keller, 1991), retinoic acid (Bain *et al.*, 1995), and TGF β 1 (Rohwedel *et al.*, 1994), have been shown to direct linear specific differentiation of mES cells. EGF and the related EGF family member, amphiregulin, are mitogenic polypeptides that induce differentiation into ectoderm and mesoderm (Gritti *et al.*, 1999). Keratinocyte growth factor (KGF) is an epithelial cell-specific mitogen responsible for normal proliferation and differentiation of epithelial cells (Visco *et al.*, 2004). In most cases, the GFs are applied to aggregates of ES cells after removal of LIF. In the absence of LIF, mES cells create intercellular contacts and initiate signaling and spontaneous differentiation (Furue *et al.*, 2005). Keller *et al.* (2004) describe the induction of epithelial- or

epidermal-specific gene expression and differentiation using a combination of GFs plus ECM components in hES cells. In our own studies, we determined that mES cells could be stimulated to differentiate and form confluent monolayers of epithelial cells with high transmonolayer resistance when grown on porous inserts coated with ECM substrata. Moreover, the inclusion of mitogenic GFs in the media, in the absence of MEF layers or LIF, could further promote differentiation (Calabro, Konsoula, and Barile, 2008, Figure 7).

ES cell cultures are model systems for studying pathways associated with tissue development and differentiation, in addition to allowing for high-throughput screening for embryotoxicity, as well as systemic, dermal, and ocular toxicity (Dholakiya and Barile, 2013). Although they are genetically a heterogeneous population, ES cells are useful in other toxicologically appropriate techniques, such as transgenesis, gene targeting, and gene trapping, which in addition, also enable the isolation of homogeneous cultures. This overcomes the limited ability of heterogeneous ES cell populations to yield ES cell-derived progenies in developmental disease models (Wobus and Boheler, 2005).

In addition to functioning as *in vitro* models for predictive toxicology of xenobiotics, stem cells are also instrumental in identification of various molecular targets (Davila *et al.*, 2004).

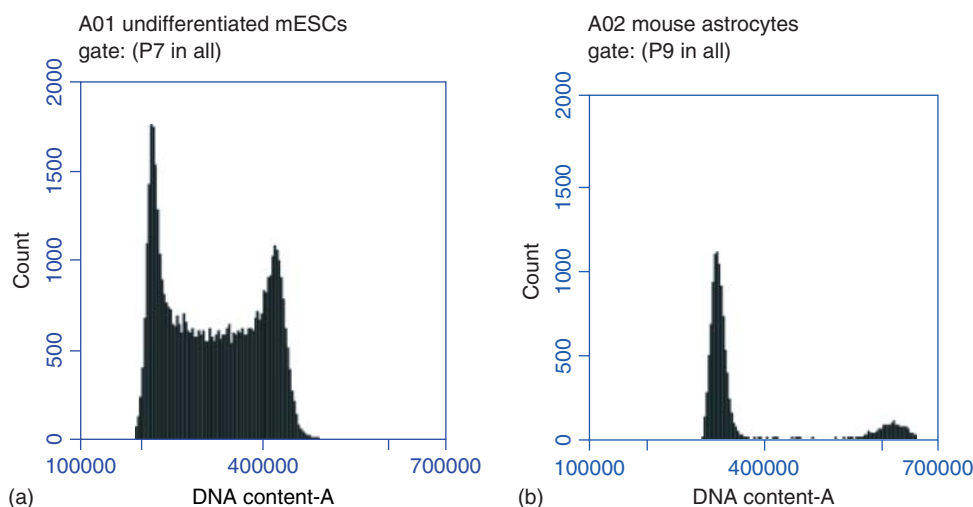


Figure 7. Comparison of cell cycle analysis of undifferentiated and differentiated mES cells. (a) mES cells were maintained in the undifferentiated state *in vitro* using LIF-supplemented growth media; cell cycle analysis was performed using propidium iodide (PI). (b) mES cells were induced to differentiate toward astrocyte lineage using growth factors.

Until recently, embryotoxicity testing was preferably performed using pregnant animals, whole embryo cultures, or embryonic cells from pregnant animals—approaches requiring extensive numbers of experimental animals. In 1997, Spielmann *et al.* (1997) proposed the potential use of ES cells *in vitro* for predicting embryotoxicity of xenobiotics, thus introducing the embryonic stem cell test (EST).

Both mouse and hES cells have been used in animal models for human diseases to study cardiac repair, nervous system repair, diabetes, and angiogenesis (see Chapter 15). However, undifferentiated ES cells form teratomas when injected at extrauterine sites, thus presenting a significant disadvantage when using stem cells for replacement therapy. Consequently, *in vivo* stem cells maintain neoplastic properties characteristic of cancer cells. *In vitro* passive exclusion of undifferentiated cells using lineage selection and tissue specificity has been attempted to overcome the formation of teratomas. However, the technique does not eliminate the current obstacles impeding the progress of therapeutic use of stem cells. Adult stem cells (ASCs), which are predominantly unipotent, are a possible alternative tool for use in stem cell therapy. Human liver, bone marrow, brain, skin, heart, and intestine have been identified as potential sources of ASCs, possessing varying levels of transcription factors (Wobus and Boheler, 2005; Chapin and Stedman, 2009). Thus, stem cells have been instrumental in the field of toxicity testing by providing an efficient alternative *in vitro* model system for animal testing and have also been simultaneously introduced as part of stem cell therapeutics.

In addition to regenerative medicine, stem cells are important tools for predicting embryonic and developmental toxicities. Until the advent of induced pluripotent stem (iPS) cells (2006), stem cells were considered to be only of either embryonic or somatic origin. Human iPS cells are reprogrammed from terminally differentiated somatic cells using several transcription factors, including Oct-4, Sox2, and Nanog. Thus, human iPS cells eliminate the need for implicating human embryos in establishing ES cells. They also reduce the risk of immune rejection upon transplantation and help in developing human cell lines of various genetic backgrounds. Furthermore, disease-specific iPS cells maintain continuous programmed characteristics. Hence, toxicology testing involving iPS cells may also be instrumental in studying other variables of toxicity

testing such as drug susceptibility, metabolism, and toxicokinetics. The cells also promise reduction of animal use in drug development and toxicity testing (Chapin and Stedman, 2009).

The striking potential benefits of stem cells in toxicology are due to their unique properties of self-renewal and pluripotency, unlike primary mammalian cells. Stem-cell-based systems offer an innovative alternative for early efficacy and toxicity screening using large numbers of cells (see Chapter 14; and Chapter 16). These properties are instrumental for improving the selection of lead drug candidates and reducing the adverse effects in later stages of drug development. For example, hepatotoxicity and cardiotoxicity are the leading reasons responsible for drug failure during pre-clinical development of drugs. Hepatocytes and cardiomyocytes derived from stem cells *in vitro* are useful for studying hepatotoxicity and cardiotoxicity of drugs early on during drug development. In addition to testing for embryotoxicity, mES cells are useful for teratogenicity screening (Genschow *et al.*, 2000; Rohwedel *et al.*, 2001; Scholz *et al.*, 1999; Spielmann *et al.*, 2001; Vanparys, 2002; Klug *et al.*, 1996).

A critical point of analysis during drug discovery for cardiotoxicity is the elongation of the QT interval, which, *in vivo*, has potential for the development of life-threatening ventricular arrhythmias. Hence, *in vitro* screening of drugs for potential effects on QT intervals is necessary before clinical trials. At present, both *in vivo* and *in vitro* models for cardiotoxicity are available, but their capacity to predict the clinical outcome is limited. Purkinje fibers are used in the establishment of *in vitro* cardiac models but are unable to accurately predict the extent of toxicity in human cardiomyocytes. Moreover, while human cardiomyocytes serve as excellent models for cardiotoxicity, their limited availability restricts their incorporation into high-throughput systems (Bird *et al.*, 2003). Alternatively, human stem cells (adult or embryonic) can be efficiently differentiated into cardiomyocytes to screen drugs for cardiotoxicity (He *et al.*, 2003; Lavon and Bevenisty, 2003). Furthermore, the ability to culture human stem cells *in vitro* enables the establishment of differentiated cardiomyocytes, particularly to replace primary cultures of human cardiomyocytes (Davila *et al.*, 2004).

All orally administered drugs undergo first-pass metabolism in liver. Hence, human liver has always

been crucial in the drug discovery blueprint. Hepatocytes extensively express hepatic enzymes responsible for phase 1 and phase 2 reactions in biotransformation and, hence, are a useful tool for studying toxicity and metabolic pathways (Raucy *et al.*, 2002; Schuetz *et al.*, 1993; Kuehl *et al.*, 2001; Lamba *et al.*, 2002). However, complete differentiation of embryonic or ASCs into hepatocytes, *in vitro*, has not been reported. In addition, there has been limited success in obtaining hepatocytes from placental-derived stem cells (PDSCs), the latter of which are capable of demonstrating mature hepatic functions as well as expressing the metabolically important CYP450 group of enzymes. As with pluripotent ES cells, PDSCs have been found to express early embryonic differentiation markers such as Oct-4 and SSEA (Davila *et al.*, 2004). Consequently, there is a formidable quest to develop robust *in vitro* systems using ES cells that are capable of not only maintaining features of pluripotency and self-renewal but also demonstrating the capacity to respond to controlled, targeted differentiation.

3.4 Influence of Stem Cells in Epigenetics

Of all live infants born, 2–5% suffer from major developmental defects, of which approximately 40% accounts for *in utero* or fetal exposure to nutritional or non-nutritional perturbations (Heindel, 2005). Conditions or disease states such as hypertension, hypoxia, diabetes, and nutritional impairment during pregnancy lead to “fetal programming.” That is, the fetus adapts to these conditions, the consequences of which may not be observed for decades. Fetal programming is not restricted to disease states but can occur from exposure to various food substances and chemicals. For example, low birth weight is consistently associated with cardiovascular diseases, depression, osteoporosis, and type 2 diabetes mellitus in the parental units (Ho and Tang, 2007). The association of low birth weight and various chronic diseases was further explained by David Barker (Calkins and Devaskar, 2011) and has been popularized as the “FeBAD” or “Barker’s hypothesis.” The theory states that during the development of an organism, there exist vulnerable periods when an organ is more sensitive to its environment, which leads to various adaptations *in utero* and in postnatal life. However, during adult periods, the plasticity toward the environment is lost, resulting

in decreased response toward environmental insults. This programming phenomenon of evolution occurs in conjunction with adaptations during fetal development, which may extend to early childhood as well. In addition, exposure to chemical toxins during an early developmental stage may serve as a critical factor in establishing the basis for acquisition of childhood or adult diseases, as toxins can affect cell growth, proliferation, and replication (Schulz, 2010; Calkins and Devaskar, 2011). In addition, while fetal exposure can lead to structural and functional alterations of the embryo, these consequences are not necessarily teratogenic, but may predispose the individual to increased susceptibility to other diseases in later life. Thus, according to the FeBAD theory, early exposure to various environmental insults with or without altered nutritional states may precipitate fetal programming without any identifiable structural abnormalities. These effects are time- and tissue-specific, often irreversible, induced by altered gene expression, genomic imprinting, gene methylation, DNA methylation, and/or chromatin remodeling—the net outcome of which is increased susceptibility to disease (see Chapter 19).

In utero or neonatal exposure to environmental agents also may set the basis for adult diseases. Furthermore, early exposure to nutritional and non-nutritional insults results in (i) occurrence of a disease that would not have occurred, (ii) increase in the risk of prevalence of the disease, or (iii) induction of the early onset of the disease or exacerbation of its effects. The consequences may also be trans-generational, hence requiring the recognition that the effects may pass through several generations (F0–F3), in order to establish an epigenetic effect. That is, *in utero* exposure (F0) to an insult results in exposure to the embryo (F1) and germ line cells (F2) (Perera and Herbstman, 2011).

In addition to the *in utero* environment, maternal–infant interactions and paternal factors, such as nutrition and aging, have been known to affect the offspring (Curley *et al.*, 2010). Epigenetic programming and reprogramming are not restricted to early stages of development but continue through the latter stages of development in response to the environmental factors (Bagot and Meaney, 2010). Furthermore, along with altered gene expression, epigenetics affects the development and function of humans from early childhood throughout the individual lifespan, as influenced by endocrine disruption, as well as other nutritional, psychological,

or environmental factors (McGowan and Szyf, 2010). During early embryogenesis, many epigenetic changes occur within the DNA domain, such as methylation, demethylation, and remethylation, which establishes genomic imprinting of genes from both maternal and paternal origins. This phenomenon, at times, results in increased susceptibility to chronic adult diseases (De Boo and Harding, 2006). Similarly, ES cell differentiation and proliferation involves various epigenetic mechanisms, such as X chromosome inactivation, which control expression of target genes. Additionally, tissue-specific genes are known to be silenced in ES cells, which reinforces the repressive state of transcriptionally inactive chromatin. In addition to stem cell differentiation, epigenetic marks also affect stem cell fate and pluripotency (Szutorisz and Dillon, 2005).

As described earlier, histone modifications involve the polycomb group of proteins to form a transcriptionally repressed environment, which maintains long-term gene silencing during development. Furthermore, PRC2 complex contains various enzymes (i.e., EED, Ezh2, Suz12), which are indispensable for murine embryonic development. For example, in hESCs, loss of EED function causes epigenetic disorders, such as Beckwith–Wiedemann syndrome.

Although the embryo undergoes frequent chromatin remodeling during early embryogenesis, the process of genomic imprinting is epigenetically stable. As the embryo demonstrates considerable epigenetic plasticity, both mESCs and hESCs are appropriate models for the study of such epigenetic changes. Further studies aiming toward epigenetic profiling of stem cells will provide better understanding of the functional relationship between phenotype, genotype, and epigenotype (Rugg-Gunn, Ferguson-Smith, and Pedersen, 2005). As mammalian ES cells have features that model early embryogenesis, the *in vitro* systems may also serve to understand the epigenetic basis of differentiation and pluripotency, as they relate to normal development and disease.

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FURTHER READING

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Use of Video Bioinformatics Tools in Stem Cell Toxicology

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1 INTRODUCTION

Human embryonic stem cells (hESCs), first derived from blastocysts in 1998 (Thomson *et al.*, 1998), provide unique cellular models with numerous potential applications (Ho *et al.*, 2012). Their adaptation to toxicological studies is particularly attractive as pluripotent stem cells can be used to model various stages of prenatal development (Adler *et al.*, 2008; Kang and Trosko, 2011; Talbot and Lin, 2011a), the period of the life cycle that is most sensitive to environmental chemicals (Barouki *et al.*, 2012; Grandjean *et al.*, 2007). The application of stem cells to toxicological work, while potentially one of their most important uses, has lagged behind other areas of development, such as their use in regenerative medicine. However, with new and better culture media and methods for performing quantitative assays with small colonies of hESCs (Behar *et al.*, 2012a; Lin and Talbot, 2011; Ludwig and Thomson, 2007), it is now feasible to begin development of more rigorous toxicological assays using hESCs.

One assay strategy is to use live cell time-lapse imaging to observe dynamic cellular processes that are affected during experimental treatment with test chemicals. The use of video microscopy in cell biology began to flourish in the 1970s and 1980s (Inoue, 1987, 1988) and has steadily improved to the point

where it has become relatively easy to perform with the introduction of new instrumentation for imaging live cells overtime (Cervinka, Cervinkova, and Rudolf, 2008; Stephens and Allan, 2003; Wollert and Langford, 2009). Incubators with built-in microscopes and robotics enable time-lapse data to be collected accurately for short- and long-term experiments. For toxicological work, it is important to be able to extract quantitative data from time-lapse videos, a difficult task because of the large size and complexity of the data. When video data analyses are done manually, many hours of personnel time may be required to complete a project, and manual analysis is subject to variation in interpretation and error. These two challenges preclude routine use of video data in toxicological work, but with the application of video bioinformatics (VBI) tools, quantitative analysis of toxicological video data is both feasible and reliable.

VBI analysis involves the use of computer software to mine specific data from video images. This method of analysis is rapid and can eliminate errors that occur when analysis is done manually. VBI tools, therefore, enable a powerful quantitative approach for extracting spatiotemporal data rapidly from video images using computer software to perform data mining and analysis. Much information can be learned by studying time-lapse data of live cells treated with toxicants. With the introduction

of commercially available VBI software packages, such as CL-Quant (Alworth, Watanabe, and Lee, 2010), it is now feasible to develop customized protocols to analyze video data and determine quantitatively how specific chemicals affect processes in either pluripotent stem cells or stem cells undergoing differentiation. This approach has not been used extensively in the past as adequate tools for collecting and mining video data have not been available until recently. In this chapter, the application of VBI tools to problems in stem cell toxicology is presented. Examples of applications will be given for pluripotent hESCs, mouse neural stem cells, and differentiating hESCs.

2 VIDEO BIOINFORMATICS BACKGROUND

2.1 What Is Video Bioinformatics and Why Is It Important?

New imaging instrumentation and devices, such as the Nikon BioStations, have become available to perform live video imaging and collect biological videos for online/off-line processing. However, data processing and analysis (informatics) techniques for handling complex biological images/videos are challenging, and until recently, most time-lapse video analysis has been done manually. Furthermore, much current automated analysis is limited to *snapshot analysis* of biological processes that are in actuality continuous and dynamic. To gain a complete mechanistic understanding of cellular

processes, it is necessary to analyze dynamic events such as cell attachment, migration, division, and apoptosis. VBI is concerned with the automated processing, analysis, understanding, data mining, visualization, query-based retrieval/storage of biological spatiotemporal events/data, and knowledge extracted from microscopic videos (www.cris.ucr.edu/IGERT/index.php). It integrates expertise from the life sciences, computer science, and engineering to enable breakthrough capabilities in understanding continuous biological processes. VBI has great promise for stem cell analysis and biological sciences in general as it can remove the subjectivity from experiments and quantify measurements precisely in a highly efficient manner. This will help in building predictive models (as opposed to qualitative models), discovering new information, mining knowledge hidden in the data, and ultimately establishing foundational principles. All of this will significantly increase the productivity of biologists and toxicologists.

2.2 Video Bioinformatics in Toxicology

VBI can aid toxicological studies in many ways. The following are some examples of dynamic end points that can be monitored and quantified in stem cells using VBI tools. Figure 1 shows control hESCs and treated hESCs that have been plated in cigarette smoke solution. The smoke has inhibited attachment and spreading of the hESCs, end points that can potentially be evaluated and quantified with VBI software. These end points can be collected

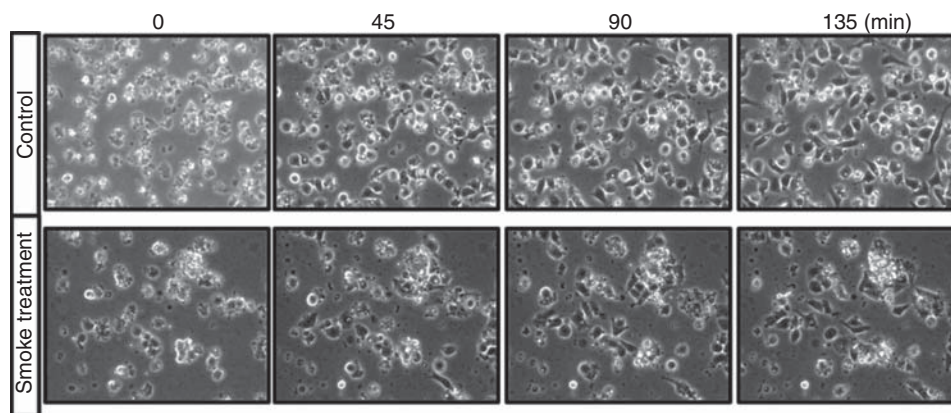


Figure 1. Cigarette smoke treatment increases cell death and decreases the number of attached cells. By 135 min of incubation, most control cells have attached and spread. In the treated group, many cells have died and fewer cells are attached and spread.

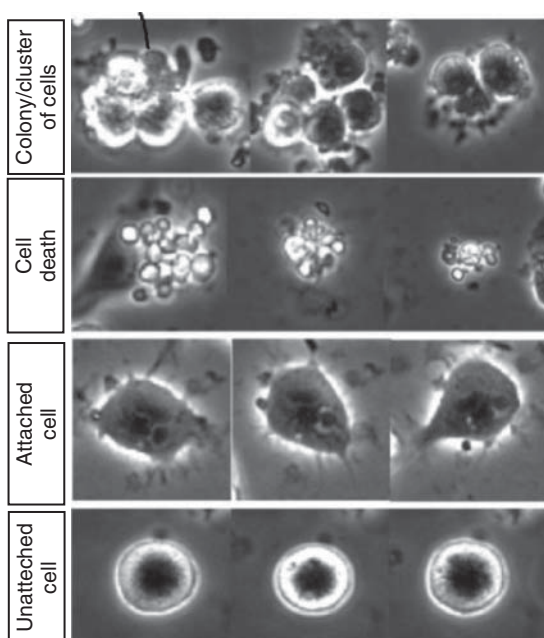


Figure 2. Micrographs showing various types or classes of single hESCs that can be distinguished using video bioinformatics tools.

in relatively short-term experiments, making them particularly attractive in toxicological assays.

Automated recognition of the state in which stem cells exist can be accomplished and quantified using VBI tools. For example, hESCs that are attached or unattached to the substrate, blebbing or spreading on the substrate, or apoptotic can be determined. This involves shape analysis, feature extraction, and classification, which then enables counting the number of cells in each state. Figures 2–4 show examples of hESCs in different states of dynamic processes.

Automated detection, segmentation, and counting of dividing stem cells in video images are also possible. hESCs typically divide mitotically every 36–48 h. VBI tools can be used to count the number of divisions. The processes occurring during division include the following: (i) the cell “stands still,” (ii) the cell divides into two daughter cells, (iii) the two daughter cells bleb, and (iv) eventually, the daughter cells stop blebbing, attach, and spread. Several of these stages of mitosis are shown in Figure 3 (frames 1–8). It is important to know how toxicants affect proliferation to determine whether a particular chemical induces a net decrease in cell

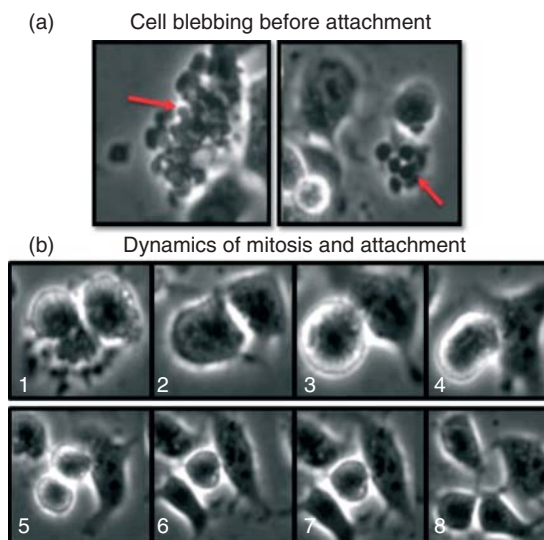


Figure 3. Micrographs of hESCs undergoing dynamic blebbing and mitosis. (a) Arrows indicate hESCs that are not attached to the substrate undergoing dynamic blebbing. (b) Sequence showing various steps that a cell goes through during mitosis.

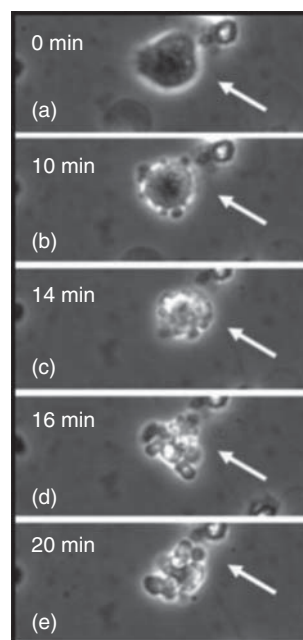


Figure 4. (a–e) Sequence of micrographs showing an attached hESC coming off the substrate and undergoing apoptotic blebbing.

number (inhibition of proliferation coupled with apoptosis).

VBI tools can also perform automated analysis for understanding and monitoring the dynamic behavior of fundamental cell processes, such as dynamic blebbing, apoptosis, self-renewal, and differentiation. For example, hESCs undergo dynamic blebbing much longer after passaging than other cell types. During dynamic blebbing, cell surfaces form and retract many small protrusions, which depend on an intact functioning cytoskeleton. Analysis of dynamic blebbing as an end point offers a method for assessing the health of the cytoskeleton in living cells in a relatively short-term experiment. Figures 2, 3 show dynamic blebbing, while Figure 4 shows blebbing in an apoptotic cell. These processes are sufficiently different that they can be distinguished, particularly when changes over time are considered.

Automated tracking of hESC colony movement across a field over time is possible and can also provide information on the health of the cytoskeleton. End points that can be monitored include distance traveled and total displacement of the colony. This analysis results in the automatic generation of a set of spatiotemporal tracks at various points, which can be further analyzed and used to detect abnormal events in biological samples.

VBI tools can be used to build dynamic statistical models of processes such as blebbing and apoptosis with or without the presence of toxicants. This provides both a foundational understanding of these phenomena and a means to know how to accelerate or decelerate the rates of these activities.

The application of VBI tools to toxicological problems using stem cells has a great potential. The entire range of dynamic cellular processes can potentially be evaluated and compared in control and treated cells. Many VBI tools have already been developed and used to solve toxicological problems, and examples of these will be presented in this chapter. New tools and integration of existing tools are currently in development.

self-renewal, they can generate more unspecialized cells. They also possess the capability to differentiate, that is, to develop into specialized somatic cells. Broadly, two classes of stem cells can be distinguished, pluripotent stem cells and adult stem cells. Adult stem cells are found in almost every organ of the adult body and contribute to tissue homeostasis, regeneration, and repair after injury. They have already committed to certain lineages. For instance, a neural stem cell found in the brain may differentiate into cell types found in the brain only, such as neurons, astrocytes, and glia. Another feature characteristic of adult stem cells is their limited proliferation potential once isolated and cultured *in vitro*. In contrast, pluripotent stem cells have an unsurpassed proliferative capacity and can undergo more than 200 population doublings (Amit and Itskovitz-Eldor, 2002).

Pluripotent stem cells are less committed than adult stem cells in that they can give rise to all of the cell types in the body. The classical pluripotent stem cell is the embryonic stem cells (ESCs), which is isolated from an early embryonic stage, the blastocyst. While murine ESC have helped toxicologists and developmental biologists uncover mechanisms of toxicity and study embryogenesis *in vitro* since their discovery in 1981 (Martin, 1981), human ESC were only isolated shortly before the turn of the century (Thomson *et al.*, 1998; Reubinoff *et al.*, 2000). Due to their human origin and pluripotency, these cells offer great promise for the development of regenerative therapies and also provide a much needed prenatal model for toxicological studies.

In addition to these two major categories of stem cells, certain intermediates share features of both classes. For example, cord blood stem cells may generate only hematopoietic cell types, but are more proliferative than hematopoietic stem cells. Amniotic fluid stem cells in turn proliferate longer in culture than adult stem cells and are broadly multipotent, that is, less restricted than adult stem cells without being fully pluripotent. Figure 5 shows examples of various types of stem cells that can potentially be used in toxicological testing.

3 STEM CELLS BACKGROUND

3.1 What Are Stem Cells?

Stem cells are unspecialized cells that give rise indefinitely to more cells. Through the process of

3.2 Why Use Stem Cells in Toxicological Studies

It is exactly two features that make stem cells what they are and are also the reasons for their use in

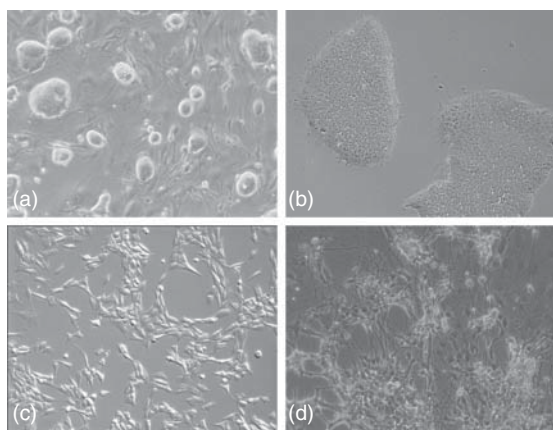


Figure 5. Micrographs of various types of stem cells and neuronal cells. (a) Mouse embryonic stem cells, line D3; (b) human embryonic stem cell, line H9; (c) mouse neural stem cells; and (d) neuronal cells differentiated from mouse embryonic stem cells according to Okabe *et al.* (1996) as described by zur Nieden, Kempka, and Ahr (2004).

toxicological studies: their proliferative nature and their development potential. Their potential use in toxicology is threefold: (i) stem cells may be used directly to study the effect of toxicants on sensitive cells, (ii) stem cells may be differentiated into a somatic cell type and subsequently be incubated with the compound of interest, and (iii) stem cells may be simultaneously differentiated and exposed to the compound of interest. While the first two are examples of classic toxicity end points, the latter is exploited to study teratogenicity, the ability of a chemical to cause developmental anomalies in a fetus. This is important as 1 in 28 babies in the United States carry congenital anomalies (March of Dimes), the bulk of which is caused by environmental influences or drugs. New chemicals and chemical by-products that are released into our environment, but are largely untested for safety, are becoming commercially available at alarming rates. A majority of these chemicals need to be comprehensively evaluated for teratogenicity using a reliable and efficient prenatal model. With appropriate data, acceptable exposure levels and actual safety of such products can then be established for individuals that are the most vulnerable to chemical exposure. Therefore, toxicology programs have been designed to identify teratogenicities that may be encountered in human embryos. *In vivo* rodent model systems and *in vitro* embryotoxicity assays, which reduce

animal testing, have been developed, such as the Micromass-Test, whole embryo culture, and the frog embryo teratogenesis assay *Xenopus* (FETAX) test. All three assays monitor defects in organogenesis in response to toxicants (Cuthbertson and Beck, 1990; Mouche, Malesic, and Gillardeaux, 2011). Although these assays allow observation of organ malformation and classification of effective teratogens, they have two drawbacks. First, they are very labor-intensive and require killing pregnant animals to recover embryos for the assays. Second, due to an elaborate morphology-based scoring system, these assays rely on subjective evaluations. Only with the use of ESCs, in the so-called embryonic stem cell test (EST), was the use of animals eliminated entirely (Spielmann *et al.*, 1997).

Before novel chemicals and drugs are certified for human use by the FDA, side effects have to be determined by the industry; this includes the aforementioned teratogenicity as well as cytotoxicity directed to organs of the person being exposed. For instance, heart, liver, and brain toxicity are the leading contributors to adverse human response and high drug attrition (Schuster, Laggner, and Langer, 2005; Guengerich, 2011). Safety tests in the pharmaceutical industry, for example, routinely include *in vitro* screening tests that examine the toxicity of novel chemicals on these tissues using genetically modified cell lines and/or primary cells isolated from rodents. Some of the drawbacks of the currently used *in vitro* assays are their low predictive potential and the low survival rate of primary isolated cells, such as hepatocytes. Therefore, the most important stem cell derivatives for toxicity screening in preclinical drug development are hepatocytes and cardiomyocytes.

3.3 Types of Assays that Have Been Done that Do Not Use Video Bioinformatics Tools

3.3.1 Assays that Use Differentiating Pluripotent Stem Cells—Teratogenicity

Examining the effects of chemicals on embryos and fetuses is important in establishing risk and to lower limits of permissible chemical exposure. Embryos and fetuses are generally more sensitive to environmental chemicals than adults. It can therefore be argued that evaluation of toxicity should be done on embryonic and fetal models, as these will usually be the most sensitive stage of the life cycle and a stage

that will suffer long-term consequences should toxicant exposure occur at this developmentally sensitive time (Grandjean *et al.*, 2007). It is also now recognized that *in utero* exposures can lead to early onset of noncommunicable diseases in adults (Barouki *et al.*, 2012), making the availability of toxicological models for prenatal development even more urgent.

ESCs from the mouse are a powerful tool for predicting general teratogenic potentials of compounds for human use. This has been demonstrated by an EU-wide evaluation study of the classic EST (Genschow *et al.*, 2004). The classical EST compares two important aspects of prenatal toxicity. First, the test determines the ability of a chemical to inhibit the differentiation of mouse ESC into contracting cardiomyocytes. Second, the EST reveals the differences in sensitivity of ESC and differentiated fibroblasts to chemical exposure. The classical differentiation end point, which is the absence or presence of contracting cardiomyocyte clusters in a given stem cell culture, has been used to evaluate the teratogenic potential of pharmaceutical compounds (Scholz *et al.*, 1999; zur Nieden *et al.*, 2001; zur Nieden, Kempka, and Ahr, 2004). In addition, molecular markers that are correlated with mature functional cardiomyocytes have been added. For example, assessing the levels of α/β myosin heavy chain mRNA or protein brings significant advantages for the *in vitro* detection of teratogenic effects by allowing earlier detection and increasing sensitivity of the assay (zur Nieden *et al.*, 2001; zur Nieden, Kempka, and Ahr, 2004; Seiler *et al.*, 2004). The benefit of using the EST over other *in vitro* assays is that it is the only current *in vitro* model that uses permanent cell lines and therefore abrogates the use of animals entirely. The disadvantage of the classic EST system is that it can only evaluate cardiogenesis and cannot be used to predict teratogenicity in other organs. However, more recently, the pluripotent nature of the cells used for the assay has been exploited to include the skeleton, the brain, and the endothelium as organ end points. This became possible because of recent advances in the field of stem cell biology, which allows directed differentiation of murine ESCs into osteoblasts (zur Nieden, Kempka, and Ahr, 2003; Phillips *et al.*, 2001), chondrocytes (zur Nieden *et al.*, 2005), neurons (Okabe *et al.*, 1996), and endothelial cells (Festag *et al.*, 2007a). Utilization of such differentiation protocols coupled with the

assessment of the tissue-specific mRNA levels in the screening of teratogens has identified chemicals with cell-lineage-specific effects (zur Nieden, Kempka, and Ahr, 2004; Festag *et al.*, 2007b). These newly introduced end points are important as they allow evaluation of cell-lineage-specific effects on diverse organs, not just the heart.

However, significant improvements to these protocols are needed to increase the reliability of predicting safety for human use. Despite the routine use of rodent models in research, the mouse model as used in the classic EST can only yield a limited understanding of human development, anatomy, and physiology. Accordingly, nonhuman primate or human *in vitro* models are desirable from a pharmacological and pathophysiological standpoint. Indeed, ESCs were established from old world (rhesus) and new world (Callithrix) monkey blastocysts (Thomson *et al.*, 1995, 1996) and are likely more predictive than mouse cells. However, it is evident that there are significant differences in the responses of nonhuman primates and humans to drugs and toxicants (Bailey, 2005). For this reason, human cells may provide the most accurate information regarding the teratogenic potential of environmental chemicals. Because it will never be feasible to perform experiments on human embryos per se, human ESC offer the best model we currently have for examining toxicants at this early stage of development.

3.3.2 Assays that Use Undifferentiated Pluripotent Stem Cells—Teratogenicity and Toxicity

As indicated previously, hESCs are derived from the inner cell mass of blastocysts (Thomson *et al.*, 1998). During *in vitro* culture, hESCs assume the characteristics of epiblast cells, which are found in postimplantation human embryos during weeks 2 and 3 of development (Nichols and Smith, 2009, 2011). hESCs, therefore, provide a very valuable model for examining the effects of chemicals on an early stage of postimplantation embryonic development. Chemicals that affect epiblast cells are likely to kill embryos or produce an effect that could lead to severe congenital defects later in development.

As an example, the relative cytotoxicity of electronic cigarette refill solutions has been determined by screening the survival of small hESC colonies

using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay, which can be done in 48 h (Bahl *et al.*, 2012). Electronic cigarettes are rapidly entering consumer markets worldwide without prior testing for safety (Hua, Yip, and Talbot, 2011; Trtchounian and Talbot, 2010; Trtchounian, Williams, and Talbot, 2010; Williams and Talbot, 2011). Results from this study clearly show that these products vary significantly in their cytotoxicity and that ESCs are, in general, more sensitive to exposure than differentiated adult cells from the lung. While additional work is necessary to fully understand the risks electronic cigarette products present to embryos and fetuses, these preliminary data are sufficient to caution women against electronic cigarette use during pregnancy. This assay makes clear that the use of pluripotent stem cells in toxicological evaluations has the further advantage of providing data rapidly. Methods have been established that enable chemicals to be readily screened in 96-well plate assays lasting from 2 h to 2 days (Behar *et al.*, 2012a, b).

3.3.3 Assays that Use Differentiated Stem Cells—Toxicity

Between 1990 and 2006, more than a third of safety-related withdrawals from the drug market were related to cardiovascular toxicity (Shah, 2006), despite the routine screening assays performed by industry. Currently, cardiotoxicity is detected using electrophysiology-based assays for interactions of compounds with potassium ion channels, but the statistics, mentioned above, emphasize how urgently cardiac toxicity screening cascades need to be substituted with more predictive assays. A number of studies have already demonstrated the applicability and the potential of stem-cell-derived cardiomyocytes, and also hepatocytes, in toxicity screening.

Cultures of contracting stem-cell-derived cardiomyocytes offer human cardiac-specific function in a cellular format with sufficient scale to support innovative new high-throughput approaches that target gaps in current cardiac toxicity screening. Human ESC-derived cardiomyocytes have been shown to be superior to rat myoblastic H9c2 cells to support structural cardiotoxicity hazard identification and to gain insight into the intricate mechanisms implicated in structural cardiotoxicity (Pointon *et al.*, 2013). Human pluripotent

stem-cell-derived cardiomyocytes could also be used to test the chronotropic activity and physiological properties of drugs. For example, using human cardiomyocytes derived from pluripotent stem cells to develop proarrhythmia assays would allow researchers to screen for proarrhythmic potential early in drug development (Nalos *et al.*, 2012). Pluripotent stem cells are also useful to uncover the mechanisms of rare heritable arrhythmias, for example the congenital long QT syndrome. Cardiac electrophysiology both for structure and for function of cardiac ion channels and their impact on heart function examined in cardiomyocytes derived from human-induced pluripotent stem cells (iPSCs) has unraveled the mechanistic basis for the resistance to therapy (Nalos *et al.*, 2012).

Similarly, human hepatocytes generated from pluripotent stem cells will allow the development of predictive hepatotoxicity screens and *in vitro* drug metabolizing assays. Hepatocytes are metabolically active cells that detoxify the body, as they are often the by-products of drug metabolism that result in toxic outcomes. Currently, human hepatocytes are utilized to test human responsiveness to new drugs. However, this remains a technically demanding approach that is limited by donor organ shortage and the tendency of cultured adult liver cells to become fetal-like. Despite some success with their isolation, it is still more reasonable to use all available donor livers for organ transplantation. This situation will remain unaltered unless an alternative to primary hepatocytes is made available. Stem cells could represent such a source. Unfortunately, the scientific community has not yet generated fully functional hepatocytes from stem cells. The hepatocyte-like cells that have been created from ESCs represent fetal hepatocytes at best. Although variety of CYP (cytochrome P450) mRNAs were identified in hESCs-derived hepatocyte-like cells and were also inducible by drugs (Ek *et al.*, 2007; Basma *et al.*, 2009), the corresponding protein was found only for CYP1A2 and CYP3A4/7. Moreover, the mRNA levels were significantly lower than in primary human hepatocytes. Once their differentiation can be successfully mastered, human pluripotent stem cells will provide the opportunity for an unlimited supply of human hepatocytes including those from specific genotypic or disease background, as well as individual patients. This is of particular importance as a drug may preferentially cause adverse events in certain individuals with a specific genetic

background or environmental history. To predict chemical response variability in humans, pluripotent stem cell lines could be made from a diverse population of individuals representing various genders, races, ages, and disease history. Perhaps, an even more powerful model of predictive toxicology could be achieved by using patient-specific iPSCs. Human iPSCs are artificially created from somatic cells of donor origin by bringing four transcription factors to expression that are associated with the pluripotent state (Takahashi *et al.*, 2007). If human iPSCs were made from individuals with known susceptibilities or resistances to various drugs or diseases or with a variety of polymorphisms in CYP liver enzymes, a wide variety of these could be used throughout drug developmental phases in conjunction with traditional *in vivo* methods, for decision making during drug selection and risk assessment.

4 INSTRUMENTATION USED TO COLLECT LIVE CELL VIDEO DATA

4.1 Incubation Directly on a Microscope Stage

If a research-grade inverted microscope with a motorized stage and camera is available, the least expensive option for live cell time-lapse imaging is placement of a small incubation unit directly on the top of an inverted microscope stage. This technology has been available for a number of years and continues to improve (Stephens and Allan, 2003; Swedlow and Platani, 2002). It involves using a small incubator that holds a multi-well plate, chamber slide, or smaller format such as a 35-mm dish. The incubator sits directly over the objective lens of an inverted microscope and is heated to maintain 37°C by an external controller unit. The CO₂ atmosphere is regulated remotely and humidity is established by placing water either in the incubator or in a remote bottle that feeds water vapor into the incubation chamber. Some units are also able to regulate O₂ levels in the incubator atmosphere, making use of hypoxic conditions feasible. It is necessary to have a motorized stage if more than one point will be imaged over time, as would usually be desirable in toxicological studies. Microscope companies have now developed methods for keeping fields of interest in focus during long-term time-lapse data collection. As an example, Nikon has introduced

the Perfect Focus System for use on their Eclipse inverted microscope line. Perfect Focus performs a real-time focus correction and can be combined with phase contrast and fluorescence imaging (Wollert and Langford, 2009).

Data can be collected using any number of objectives and at different magnifications. Excellent high-quality images can be obtained at any magnification by optimizing the objectives to the type of dish and the cells being studied. Phase contrast or Hoffman modulation objectives can be used with plastic tissue culture dishes, while DIC (differential interference contrast) would be preferable for glass dishes or chamber slides. Incubating cells in 24 or 48 multi-well plates is particularly useful in toxicological studies that require different doses be tested. However, imaging in 96-well plates sometimes produces too much distortion of the image to be useful. When using 35-mm dishes, the control and treated groups are usually run at different times, perhaps using different passages of cells, thereby making the experiment less controlled. For toxicological studies, the use of a plate with multiple wells facilitates work and minimizes variables.

A number of companies manufacture stage top incubators. The one shown in Figure 6 is made by Pathology Devices, and we have had good results with this unit. Tokai Hit also makes a well-respected product. The stage top incubator plus controller costs between \$17 000 and \$25 000 depending on the manufacturer and hardware. The Pathology Devices system shown in Figure 6 operates on a Nikon Eclipse inverted microscope with a motorized stage, Perfect Focus System, and high-resolution CCD (charge-coupled device) camera. The microscope hardware is controlled by the Nikon Elements software package, which also performs acquisition and some types of analysis. Nikon Elements is also able to perform deconvolution of images either on-the-fly or as a postprocessing step (Figure 9).

4.2 BioStation IM

During the past 7 years, a number of companies have introduced incubators that contain built-in microscopes and cameras for the purpose of collecting data on living cells in an environment that is stable and optimal for cell culture. In retrospect, it is surprising that this type of instrumentation was



Figure 6. A Nikon inverted microscope with a Pathology Devices stage top incubator: (a) motorized stage, and high-resolution camera. The microscope hardware is integrated through Nikon Elements software. The stage top incubator is controlled through an external unit (b) that regulates humidity, temperature, and CO₂. A small stage top incubator coupled to an external control unit can provide a stable environment for live cell imaging over hours or days.

not introduced earlier as it is very versatile and convenient, and it generally produces high-quality images with minimal stress to cultured cells. This type of instrumentation comes in a variety of sizes and prices and the costs can vary significantly. For laboratories doing extensive time-lapse or video work, investment in a self-contained live cell imaging system will pay off.

The BioStation IM or its newer version, the IM-Q, manufactured by Nikon, is an example of a small benchtop self-contained incubation unit with a built-in high-sensitivity cooled CCD camera and software for controlling exposures, objectives, and the type of imaging (e.g., phase contrast or fluorescence) (Figure 7). The components of this instrument are fully integrated and easy to learn to use. Cells are maintained at a constant temperature and relative humidity in a 5% CO₂ atmosphere. The BioStation IM/IM-Q images in both the phase contrast and fluorescent mode features a no focus drift design that enables time-lapse data to be collected reliably over hours or days. Imaging can be performed in the X, Y, and Z direction. The unit comes with a well-designed software package with a user-friendly GUI (graphical user interface) for controlling the instrument and all experimental

parameters. The BioStation IM has a small footprint that conveniently fits on a benchtop. The cost is reasonable and may be less than a stage top incubator if the appropriate microscope is not available. This unit can be purchased with a perfusion option (Figure 7d), a feature that can be useful in toxicological work.

The BioStation IM-Q is available in two models, optimized for either glass bottom or plastic bottom culture dishes. Thought should be given to the type of imaging needed to select the proper instrument. Both models accommodate 35- and 60-mm culture dishes, although the magnification range is somewhat different in the two models. A four-chambered culture dish, the Hi-Q4 sold by Nikon, can be used for examining different conditions of culture in the same experiment (Figure 7e). This type of dish is especially useful for toxicological work in which more than one dose of a test substance is examined. The instrument comes complete with powerful easy-to-use software.

The BioStation IM has been recognized as a potentially valuable type of instrumentation for toxicological work (Cervinka, Cervinkova, and Rudolf, 2008) and has been directly applied to mechanistic studies of toxicology in which live cell imaging was needed (Pust *et al.*, 2010; Rudolf

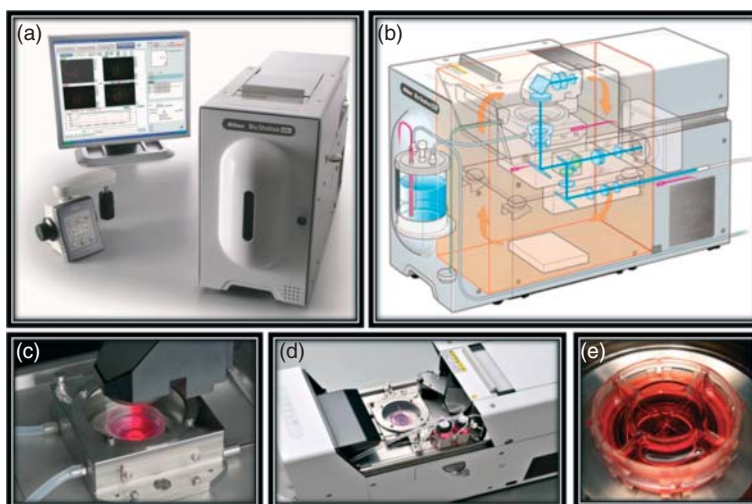


Figure 7. The Nikon BioStation IM-Q benchtop live cell imaging system. (a) External features include incubation unit, joystick for controlling the position of the camera during field of interest selection, and monitor (computer is not shown). (b) Phantom view showing the internal features of the BioStation IM-Q including air flow and sensors plus optical path. (c) Culture dish sitting inside the BioStation IM-Q incubator unit. (d) BioStation set up with perfusion attachments. (e) Hi-Q4 dish sold by Nikon, which provides four separate chambers for testing different parameters in one dish. This dish is especially useful in toxicological studies.

and Cervinka, 2010; San Miguel *et al.*, 2010), as well as to toxicological studies using stem cells (Lin *et al.*, 2010a). Although not geared for high-content or high-throughput screening, BioStation IM technology can certainly be applied to small-scale toxicological problems with excellent results.

4.3 BioStation CT

Nikon also manufactures a fully integrated high-content BioStation CT that performs time-lapse live cell imaging on up to 30 different plates or dishes at one time (Figure 8). This instrumentation is ideal for long-term cultures, which would include toxicological studies on differentiating cells, and it can also be used in short-term experiments with pluripotent stem cells. The high capacity of the BioStation CT makes it ideal for core facilities where multiple experiments may be done by different investigators simultaneously. Various types of cell containers can be placed in the BioStation CT including 6–24 multi-well plate formats, flasks, and 35- or 60-mm dishes. Different types of vessels can be used simultaneously. Plates with 24 wells are ideal for dose-response experiments in toxicological or drug testing assays. The environment

within the BioStation CT is managed and logged so that any changes in temperature, humidity, and CO₂ concentration can be detected. This unit contains two cameras, a color camera for collecting an overview of the culture plates and any labeling on the plate and a high-resolution black-and-white camera for collecting phase contrast and fluorescent images of cells (Figure 8).

The BioStation CT houses up to 30 plates in a stocker that interfaces with a robotic arm. The robotic arm transfers plates or dishes to the microscope stage for imaging at predetermined times. The user can either select the field for time-lapse imaging or allow the BioStation to select five points in a predefined pattern. Additionally, the ability to automate acquiring every available field of view in a well is possible; a function known as *full well scanning*. Higher magnifications of the full well scanning option are integrated in the software allowing the research to inspect specific cell or colony regions with higher resolution over time. The registration between robotic transfers is excellent, and transfers between the stocker and microscope can be done relatively quickly. Plates are imaged by a microscope with multiple magnifications available. The microscope can image in phase contrast or fluorescent modes. The instrument has useful features for versatile incubation, such as

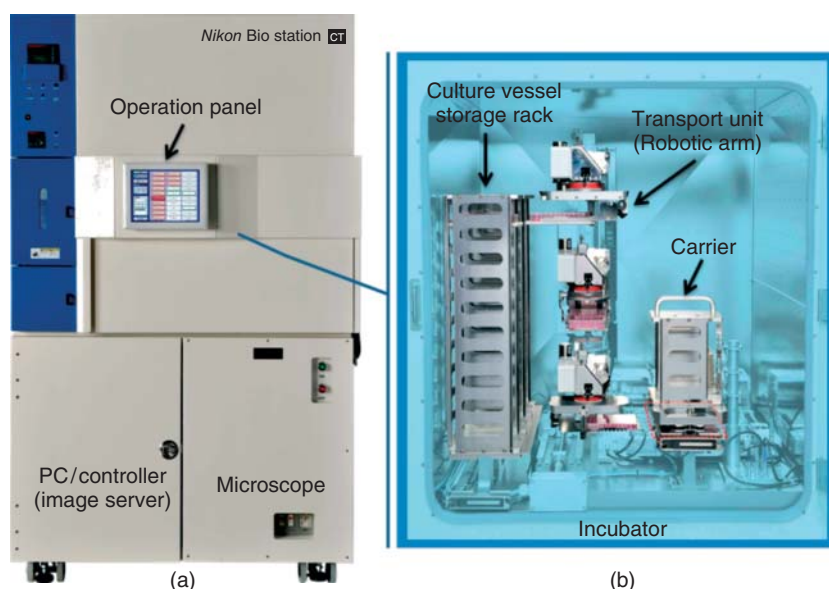


Figure 8. Nikon BioStation CT high-content live cell imaging system. (a) The BioStation CT is controlled externally by the touch screen operation panel that enables scheduling of imaging and viewing of cells. (b) The large incubation unit houses a culture vessel storage rack that holds as many as 30 culture vessels, a robotic arm, carrier, and microscope stage (not visible—behind carrier). The microscope, camera, and PC controller are located in the lower section of the instrument. A second camera enables a macro view of the vessel when it is in the microscope position.

the use of low oxygen atmospheres, and imaging, including the ability to record the entire field in a 6-well plate and stitch images together into a single field. The BioStation CT can be purchased with CL-Quant software (Section 5.2) for performing quantitative VBI analysis of time-lapse data. Because of the numerous plate options for incubation, this instrument is especially well suited for toxicological screens or dose-response experiments (Lin *et al.*, 2010a). Moreover, the ability to perform long-incubation experiments in the BioStation CT enables toxicological work on differentiating stem cells to be done easily.

Examples of the application of a BioStation CT to toxicological problems involving stem cells are presented in later sections.

4.4 Other Considerations

When setting up to collect video data on living cells in toxicological experiments, many types of end points are possible. For example, movement of whole cells or colonies of cells can be studied at relatively low resolution, while movement of organelles

or proteins within cells would require higher magnification and resolution. These factors should be considered when acquiring new instrumentation or setting up an experiment. Video images, especially of fluorescent fields, may benefit from image enhancement such as deconvolution. Nikon Elements software, for example, can deconvolute video data on-the-fly or during postacquisition processing. Figure 9 shows the zona pellucida of a hamster oocyte stained with an antibody to a zona pellucida protein before and after deconvolution, which has improved image quality.

5 SOFTWARE USED FOR VIDEO BIOINFORMATICS ANALYSIS OF STEM CELLS IN TOXICOLOGICAL APPLICATIONS

5.1 General Considerations

For most toxicological applications with stem cells, video data will need to be analyzed quantitatively. This is challenging due to the vast amount of data

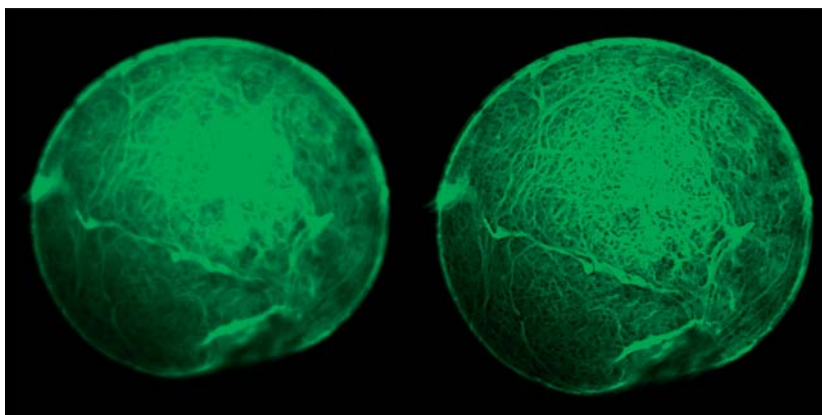


Figure 9. Unprocessed and deconvoluted image of the zona pellucida around a mouse oocyte. The zona has been labeled with an antibody to ZP3. In the unprocessed image, noise detracts from the resolution. Following deconvolution, numerous fibers are apparent in the zona. Deconvolution of time-lapse images can be done on the fly using Nikon Elements software.

that can be obtained and the complexity of the data. Application of software tools to such analysis not only saves time but also enables more accurate reliable data and interpretation of videos. Computer analysis often reveals information that humans do not detect due to the complexity of the information contained in videos. Some commercial software tools are available for video image analysis and several of these will be discussed. Custom software tools can also be developed and used for specific problems or complex problems that may require building up multiple levels of analysis for a single experiment. Such software is currently in development in our IGERT (Integrative Graduate Education and Research Traineeship) VBI program and examples will also be given in a later section.

5.2 CL-Quant Software

CL-Quant software is a live cell image analysis program produced for Nikon by DRVision Technologies (Bellevue, Washington). It can be purchased with BioStation hardware or the same package can be purchased directly from DRVision under the name SVCell. CL-Quant provides tools for developing protocols for recognition and quantitative analysis of microscopic data, including video data (Alworth, Watanabe, and Lee, 2010). It can be applied with user-directed learning and does not require image processing knowledge. The

current version of the software is user-friendly and comes with webinar instruction. CL-Quant can be used to detect, segment, measure, classify, analyze, and discover cellular phenotypes in video data. It can be used with both phase contrast and fluorescent images. Preconfigured modules are available for some applications such as cell counting, confluency, and cell division. However, the software has significant depth and can be configured for other applications by the user. Later in this chapter, we will show an example of adapting CL-Quant to measure hESC colony size in time-lapse videos.

5.3 Image J

Image J is a highly versatile free software package inspired by the National Institutes of Health (NIH) Image for the Macintosh. It is written in Java, compatible with major operating systems (Linux, Mac OS X, and Windows), works with 8-, 16-, and 32-bit images, and has open software architecture that enables the use of plug-ins. It is able to read numerous file formats, and it supports “stacks.” Image J is able to perform numerous standard image processing operations that may be useful in laboratories dealing with VBI. For example, it can convert tif images to the avi format, and it can be used to obtain ground truth information when setting up a new VBI protocol. Full information about Image J is available at <http://rsb.info.nih.gov/ij/>.

5.4 MatLab

MatLab is an image processing toolbox commonly used for undergraduate and graduate courses in image processing and analysis and for rapid prototyping (www.mathworks.com/products/image/). It does not have any programs that can be directly used for stem cell analysis. However, one can assemble programs for useful purposes using the basic functions that are provided in MatLab.

5.5 Farsight

Farsight has developed quantitative tools for studying complex and dynamic biological microenvironments from 4D/5D microscopy data. It is a toolkit with a set of software modules and standardized interfaces (www.farsight-toolkit.org/wiki/Main_Page). It does not have a specific package for stem cells but some of the programs may be useful in stem cell analysis.

5.6 BioSig

BioSig has been developed at the Lawrence Berkeley National Laboratory for phenotypic analysis. It pioneered the application of information technology to the biological sciences, but it is largely directed at helping scientists manage their existing collections of data and images (Parvin *et al.*, 2003).

6 APPLICATIONS TO PLURIPOTENT STEM CELLS

6.1 Establishing and Validating a Video Bioinformatics Protocol for Measuring Colony Growth

Prior to performing VBI analysis on time-lapse data, it is necessary to have a protocol for performing the operation of interest. In this section, the development of a protocol for quantifying growth of hESC colonies will be described (Lin *et al.*, 2010b). This protocol was developed using the CL-Quant software package and videos of H9 hESC colonies taken with a BioStation CT.

The purpose of this study was to collect time-lapse data on growing hESC colonies and develop and

evaluate a software protocol that could accurately quantify hESC colony growth over a 48-h period. H9 hESCs were seeded on 12-well plates coated with Matrigel and allowed to attach for 48 h. They were then placed in a BioStation CT for an additional 48 h during which time images were taken at five different locations in each well at 7-min intervals. Examples of colonies at various times of incubation are shown in Figure 10a–c. Details of the setup procedure can be found online (Lin *et al.*, 2010b).

CL-Quant software was used to create a protocol for quantifying colony area in number of pixels. The protocol was applied in sequential steps to video data of hESCs incubated using control conditions. When applying the CL-Quant software protocol, the first step segmented the image to select mainly the hESC colony (Figure 10d–f). In the second step, the image was enhanced to remove any noise or small particles that were masked during segmentation. In the third step, the actual measurement of colony size was made. The protocol was applied to all images in five time-lapse videos to obtain growth curves for each colony over 48 h. Figure 10g shows the raw data obtained in this analysis. The ground truth analysis done using Adobe Photoshop is also shown in Figure 10g. The ground truth (dotted line) and CL-Quant-derived growth rates are very similar for each colony. Because colonies were of different sizes at the onset of the experiment, the data were normalized and replotted as a percent change over time (Figure 10h). Again, the ground truth (dotted lines) and the CL-Quant-derived values are in very good agreement. All colonies showed a slight decrease in size at the onset of the incubation followed by similar growth rates over the latter two-thirds of the incubation period. The normalized data further show that growth rates were not affected by the starting size of the colony. Figure 10 shows the means for the five colonies determined from the CL-Quant and Photoshop (ground truth) analysis. Growth curves obtained with both software packages show excellent agreement, thereby validating the protocol developed using CL-Quant software. In any protocol or software development for VBI applications, it is important to always establish a data set containing ground truth to which analysis with the developed protocol can be compared, as shown in the preceding example.

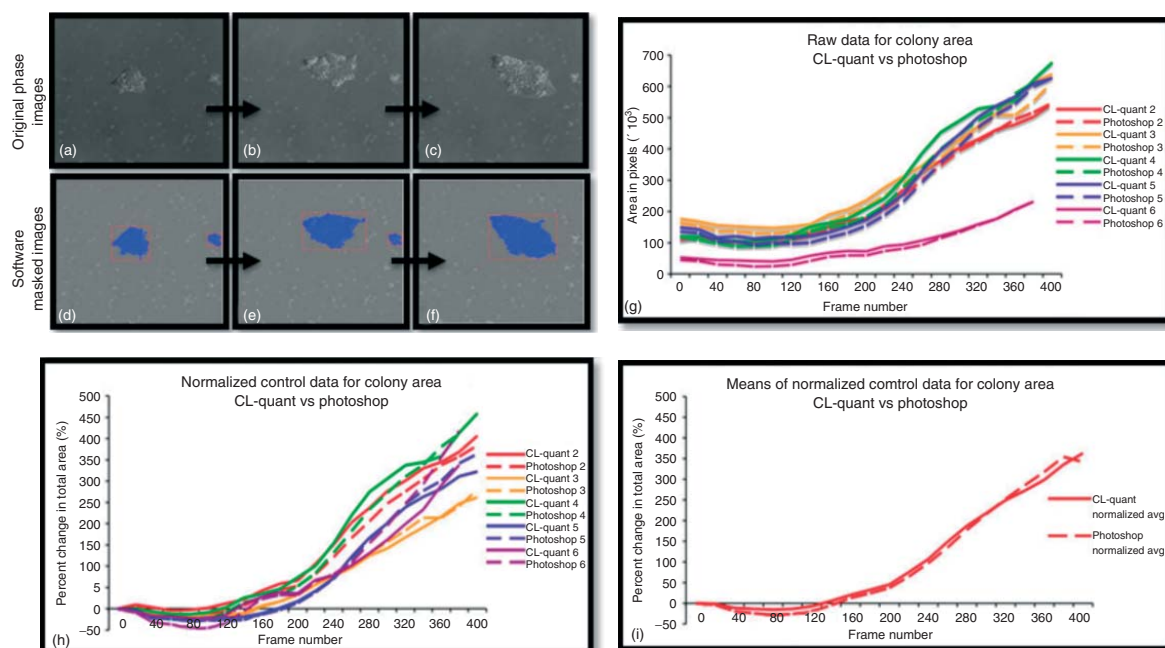


Figure 10. Development of a protocol for measuring hESC colony growth. (a–c) Phase contrast image film strip of a hESC colony showing frames three times during 48 h of incubation in a BioStation CT. (d–f) The same three images following segmentation with CL-Quant software to isolate the hESC colony. One feature in the background has also been masked, indicating further processing is needed. (g) Graph of raw data showing the increase in colony size (in pixels) over 48 h as determined using CL-Quant software (solid lines) and Photoshop (dotted lines) (ground truth). (h) The same data normalized to the area at time zero to show the percent increase in colony size over 48 h. (i) Graph showing the means for the normalized data in (h). This graph clearly shows good agreement between the analyses done using the CL-Quant software and the ground truth obtained with Photoshop. (Lin, S, *et al.*, (2010b) Reproduced with permission from Journal of Visualized Experiments.)

6.2 Application of the Video Bioinformatics Protocol for Cell Growth to a Toxicological Problem Using Stem Cells

The above protocol for quantifying colony growth was used to compare the potency of cigarette smoke from conventional and harm reduction cigarettes on growth of hESCs (Lin *et al.*, 2010a). Harm reduction cigarettes are often marketed as less toxic than conventional brands (Hatsukami *et al.*, 2007). However, relatively few tests of this concept have been published, and research on model systems (Lin, Tran, and Talbot, 2009; Riveles *et al.*, 2007) as well as data from human smokers (Shields, 2002; Warner, 2005) have shown that harm reduction products may be as dangerous as conventional tobacco products. Cigarette smoking is correlated with increases in reproductive problems, including adverse effects on the female reproductive system and the embryo/fetus (Dechanet *et al.*, 2011; Prelog, 2011; Talbot, 2008; Talbot and Lin, 2011b). In a study using mouse ESCs, which did not take advantage of VBI tools for analysis of time-lapse data, significant toxicity was in smoke from harm reduction products (Lin, Tran, and Talbot, 2009). In a subsequent study using hESCs, the effects of both mainstream (MS) and sidestream (SS) smoke on hESC colony growth were compared to untreated controls using VBI analysis (Lin *et al.*, 2010a). MS smoke is inhaled by active smokers, while SS smoke burns off the end of a cigarette (EPA, 1992; Knoll and Talbot, 1998). SS smoke is potentially more harmful than MS smoke as it burns at a lower temperature (EPA, 1992), and in numerous studies, SS smoke has been more potent than its MS counterpart (Gieseke and Talbot, 2005; Knoll and Talbot, 1998; Lin, Tran, and Talbot, 2009; Riveles *et al.*, 2007; Schick and Glantz, 2005). Moreover, some cigarettes products that have claimed to be harm reduction products do not necessarily reduce harm. For example, “light” cigarettes were marketed as having lower tar and nicotine yields; however, as was learned after they had been used many years, smokers compensated for lower nicotine by puffing longer and harder and smoking more frequently, which negated their harm reduction benefit (Gartner and Hall, 2010; Zeller *et al.*, 2009). Another harm reduction strategy has been to lower the level of carcinogens and known toxicants in cigarette smoke either by using filters, diluting the smoke with ventilation holes in the

cigarette paper, or modifying the curing procedures used to prepare tobacco (Hatsukami *et al.*, 2004; Shields, 2002).

In the study to be presented, cigarette manufacturers attempted to reduce harm reduction by using complex filters (Marlboro Lights and Advance Lights) or by genetically modifying the tobacco to produce less nicotine (Quest). The overall approach used to compare the effect of conventional (Marlboro Red) and harm reduction MS and SS smoke on the growth rates of hESC colonies is shown in Figure 11. Colonies containing 5–8 cells were plated on Matrigel-coated 12-well plates using mTeSR medium. When confluency reached about 30–40% (usually by 48 h after plating), medium was replaced with either control medium (mTeSR alone) or mTeSR containing 0.1 puff equivalent (PE) of MS or SS smoke. A PE is the amount of smoke in one puff that dissolves in 1 ml of medium. A dose of 0.1 PE was chosen as this had previously been shown to not kill cells. Plates were then incubated in the BioStation CT for 48 h during which time phase contrast images of five colonies were collected in each well every 140 min. Images were downloaded from the BioStation server and analyzed using CL-Quant software with the custom-developed algorithm described in the preceding section. This analysis included (i) an initial segmentation code that utilized a channel mask to separate the colony and background in each image, (ii) an enhancement code that filtered out any remaining image noise (e.g., cellular debris or foreign particulates), and (iii) a measurement code that quantified the number of pixels in each colony. Data obtained with the CL-Quant analysis were plotted and analyzed to determine growth rates and percent increase in size of colonies over 48 h. The accuracy of the CL-Quant protocol was verified by periodically checking the fit of masks and by rerunning data to assure reproducibility.

Colony growth was analyzed using the CL-Quant algorithm described in the preceding section for hESC colonies treated with MS or SS smoke from a conventional cigarette (Marlboro Red) and three harm reduction brands of cigarettes (Marlboro Light, Advance Light, and Quest) (Figure 11). Treatment with 0.1 PE of MS smoke from the four test brands of cigarettes did not alter the growth rate of colonies when compared to the untreated control (Figure 11a). Moreover, when analyzed by

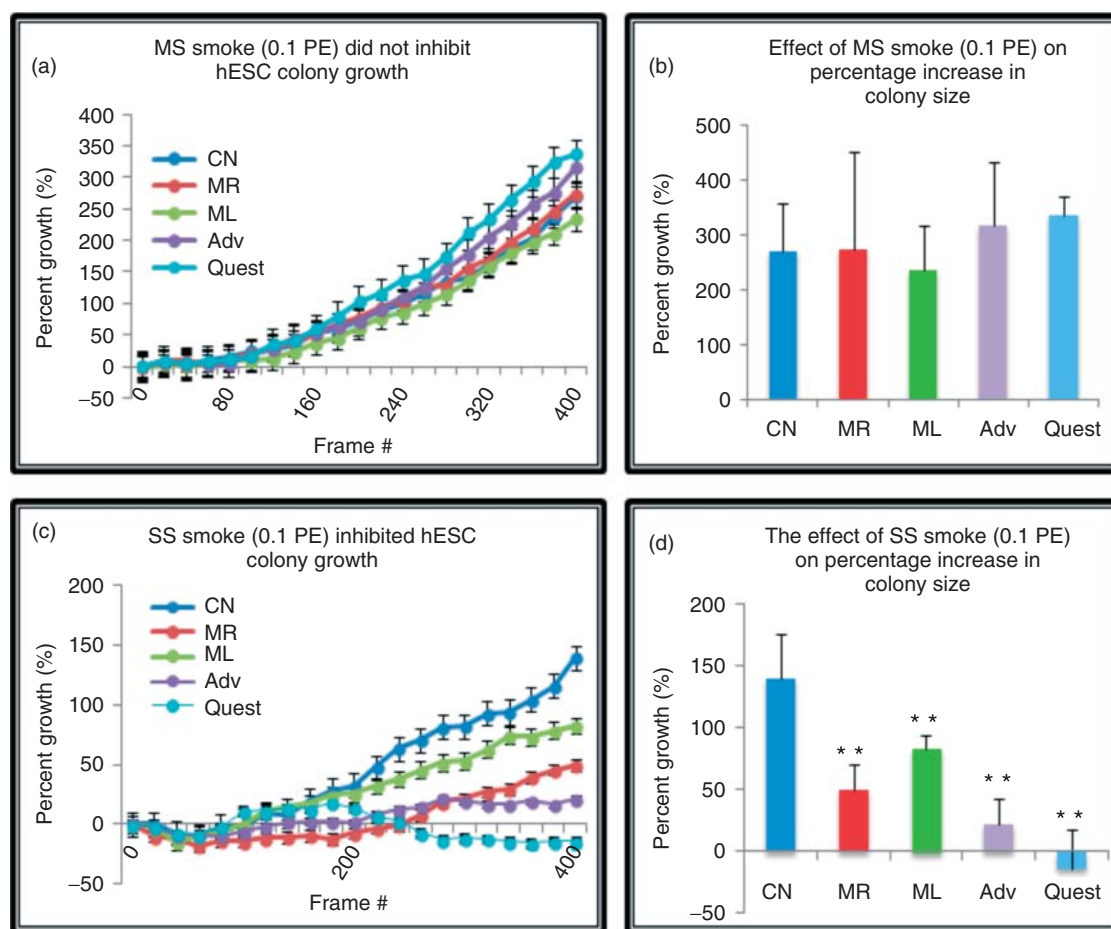


Figure 11. Growth of hESC colonies in control and treated groups (0.1 PE of mainstream or sidestream smoke solution) during 48 h of incubation in a BioStation CT. (a) Growth rates of control and mainstream (MS)-treated colonies. (b) Percentage increase in colony size at 48 h for control and mainstream-treated colonies. (c) Growth rates of control and sidestream-treated colonies. (d) Percentage increase in colony size at 48 h for control and sidestream-treated colonies. For (a) and (c), values are plotted as means $\pm$ SEM. For (b) and (d), values are plotted as means $\pm$ SD. All means are based on four separate experiments, except for Quest that was based on three experiments. In each experiment, 4–10 colonies were analyzed. Analyses were done using CL-Quant software. PE is the amount of smoke in one puff that dissolves in 1 ml of medium. CN, control; MR, Marlboro Red; ML, Marlboro Light; Adv, Advance Light; Quest, Quest no nicotine. (Lin, S, *et al.*, (2010b) Reproduced with permission from Journal of Visualized Experiments.)

ANOVA (analysis of variance), the mean percentage increase in colony size at the final frame was not significantly different among MS-treated groups ($p=0.79$) (Figure 11b). In contrast, SS smoke from all brands tested inhibited hESC growth relative to the untreated control (Figure 11c). All groups showed a small decrease in colony size during the initial phase of incubation (Figure 11c). Controls recovered from this decrease more rapidly than the treated groups. The mean percentage increase

in colony size at 48 h was significantly lower in all treated groups than in the control (Figure 11d). SS smoke from Quest and Advance, both harm reduction products, was the most potent of the four brands tested in the colony growth assay. Quest-treated colonies showed a net decrease in size, probably because of loss of cells from the colony by apoptosis, as well as failure of the surviving cells to divide as frequently as in the control group.

6.3 Application of the Gap Closure Assay to Stem Cells Using Video Bioinformatics Tools

The gap closure assay, also called the *scratch* or *wound healing assay*, has been used for many years to quantify cell migration into a gap created in a monolayer of cells (Liang, Park, and Guan, 2007; Rodriguez, Wu, and Guan, 2005). It is a simple, straightforward, and economical method for quantifying migration of cell populations. It can be performed in most laboratories set up for cell culture. VBI tools can be applied to the gap closure assay to determine the effects of treatment on the rate of cell migration.

The principle of the gap closure assay is simple. Cells are grown in a monolayer and a scratch is made through the cells in such a way as to remove a strip of cells from the center of a confluent area. The migration of cells back into the gap can be measured over time and serves as a convenient method to assay cell migration in a two-dimensional model. We have not been able to use this assay with pluripotent stem cells that grow in colonies or with cells that require Matrigel for attachment, as these cells tend to detach in sheets when the scratch is made. However, we have successfully applied this assay to mouse neural stem cells that grow as single cells and do not require Matrigel for attachment. The time to perform the assay is relatively short. Mouse neural stem cells require 1–2 days to reach confluency and another 24–48 h is needed to close the gap.

As shown in Figure 12, mouse neural stem cells were grown in Ibidi gap closure inserts. Once the cells reached confluency, the insert was removed by creating a uniform gap between two populations of cells. Mouse neural stem cells were then treated with solutions of SS smoke from various brands of conventional cigarettes. Time-lapse images of the gaps were collected over a 24-h period and then processed using VBI tools developed with CL-Quant software (Figure 12a). The area in the gap was masked and quantified in pixels over time. Quantitative analysis showed that each cigarette brand slowed the rate of gap closure relative to the untreated control (Figure 12b). Statistical analysis at the 8-h time point revealed that two brands of cigarettes (Marlboro Red and Marlboro Lights) significantly slowed gap closure (Figure 12c). Moreover, the time to gap closure was longer in all three treated groups than

in the control (Figure 12d). These data demonstrate an inhibitory effect of SS smoke solution on the motility of mouse neural stem cells.

7 APPLICATION TO DIFFERENTIATING STEM CELLS

7.1 Osteogenesis—Calcium Deposition

Although predictive for teratogenic effects on skeletal tissues, measuring mRNA levels of bone-specific genes by quantitative PCR (polymerase chain reaction), which is an end point in the EST as presented above, is very costly. The high costs associated with the modified molecular assay have precluded its routine use in laboratories around the world. Efforts are therefore being undertaken to develop novel end point assays that may save cost and time. For instance, we have shown that using an improved plating technique to evenly distribute cells can substantially decrease the time that is needed to complete the assay (zur Nieden and Baumgartner, 2010).

Moreover, the determination of calcification yield using morphometric approaches proved useful to detect skeletal teratogenicity *in vitro* (zur Nieden, Davis, and Rancourt, 2010a,b; zur Nieden and Baumgartner, 2010). Gray pixel values are assigned to static pictures of osteogenic cultures, in which the calcified areas appear black (zur Nieden *et al.*, 2007). Compared to non-treated cultures, a degree of blackness can be calculated using Image J software that corresponds with the degree of teratogenicity in treated cultures. We consider these 'macro'molecular approaches a significant advancement to prior variants of the assay, as costs that are normally incurred by molecular or biochemical assays can be substantially lowered.

While the morphometric analysis of static images has proven useful for determining end point toxicity, VBI may be used to trace calcification in a treated culture over time. For example, VBI has been used to evaluate the teratogenicity of cigarette smoke solutions on skeletal development using human ESC. H9 ESC were induced to undergo a previously developed osteogenic differentiation protocol using an overgrowth method and supplementation of medium with the osteogenic factors $1\alpha,25\text{-OH}_2$ vitamin D3, ascorbic acid, and β -glycerophosphate (modified from zur Nieden, Kempka, and Ahr,

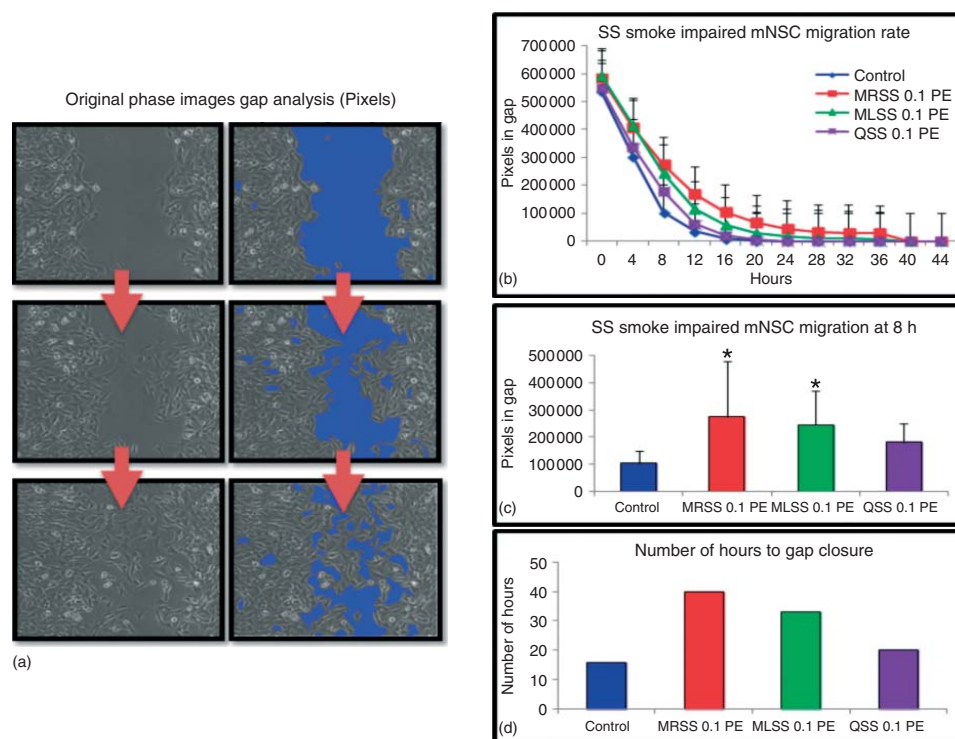


Figure 12. Effect of sidestream smoke treatment on the rate of gap closure using mouse neural stem cells. (a) Original phase contrast images and corresponding segmented images in which the gap has been masked. (b) Rate of gap closure for control and treated groups over 44 h. (c) Size of the gap in pixels at the 8-h time points. Both the Marlboro Red and Marlboro Light sidestream smoke-treated groups are closing the gap more slowly than the untreated control. (d) The number of hours to gap closure for the control and treated groups. *, $P < 0.05$; MR, Marlboro Red; ML, Marlboro Light; Q, Quest; SS, sidestream smoke.

2003). Simultaneously with the differentiation induction, cells were treated with various doses of cigarette smoke solution, and cultures were maintained for a period of 20 days. Time-lapse images were obtained using the BioStation CT. Each cell line was represented by three replicates; each comprised of 10 time-lapse videos with images taken every 12 h (Figure 13). Calcification due to emergence of mature osteoblasts was segmented using a manual thresholding algorithm (pixels with values < 33) in Matrix Laboratory (Figure 13a, bottom panels) and charted over time (Figure 13c). Similar to a biochemical analysis, which quantifies

the amount of calcium ions deposited into the extracellular matrix, calcification analysis via VBI was able to determine the difference between a mild teratogenic and a severe teratogenic dose of smoke solution (Figure 13b). This demonstrates the capability of the program to segment areas of calcification from phase contrast images of the time-lapse series.

In addition, VBI tools can be exploited to calculate the difference in calcification kinetics, that is, the amount of calcium added over a given period of time (Figure 13d). This type of analysis clearly shows that there is a delay in the onset of calcification

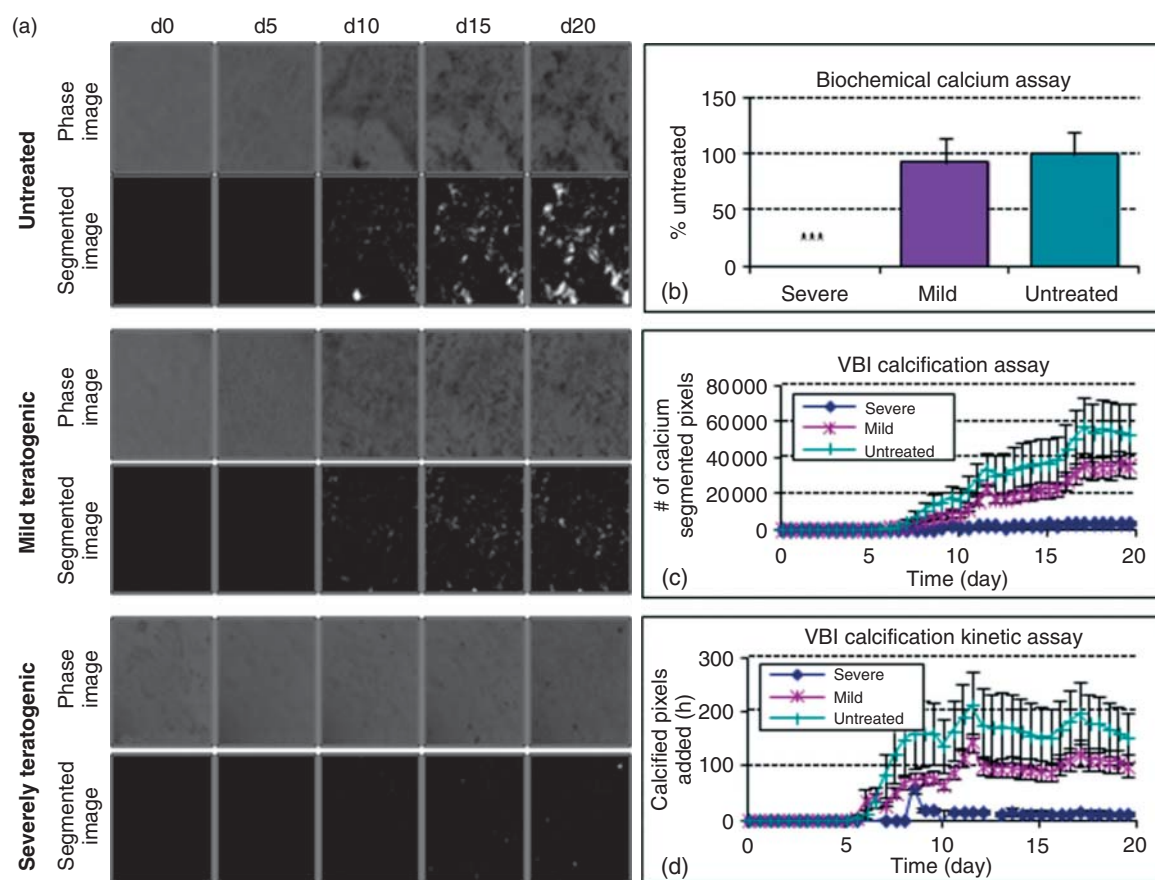


Figure 13. (a) Time-course phase contrast images of osteogenic cultures, either untreated or treated with mild teratogenic (0.003 PE) or severe teratogenic doses (0.3 PE) of sidestream (SS) Marlboro Light smoke solution. Representative images were chosen from a collection of images taken every 12 h over a period of 20 days using a Nikon BioStation CT and are shown in the respective top panels. Respective bottom panels represent segmented images using video bioinformatics software designed to measure levels of calcification in osteogenic cultures from time-lapse videos. (b) End point calcium measurement using a reagent-based assay, Arsenazo III, at d20 of osteogenic differentiation. *** $p < 0.05$, one-way ANOVA, $n = 5 \pm \text{SD}$. (c) Calcification of mild and severe teratogenic cultures using video-based analysis, $n = 30 \pm \text{SEM}$. (d) Calcification rates of mild and severe teratogenic cultures as measured using video-based analysis and represented as number of segmented calcified pixels added per hour, $n = 30 \pm \text{SEM}$.

in the severely teratogenic dose at approximately day 7 of differentiation, from which the cells never recover. In this analysis, therefore, teratogenicity may be established at earlier time points of the *in vitro* assay, which would reduce overall time and cost of such assays.

8 ANALYSIS, COMPUTATION, AND FUTURE DIRECTIONS

8.1 Analysis and Computation

The following are several examples for the automated detection of stem cells that have been developed in the IGERT VBI program at UCR (Guan *et al.*, 2011, 2012a,b, 2013). These methods are being developed for several purposes that include their eventual use in toxicological assays. Figure 14 shows the automatic detection of non-dynamic blebbing single unattached human embryonic stem cells (NDBSU-hESCs). The approach uses multiple trained classifiers where each classifier eliminates cells with properties unmatched to NDBSU-hESCs. The video data were collected using the BioStation IM. The video frames were taken using an objective of 20 with a 600×800 pixel resolution. Each frame is captured every 10 min. The automatic method yields a high true

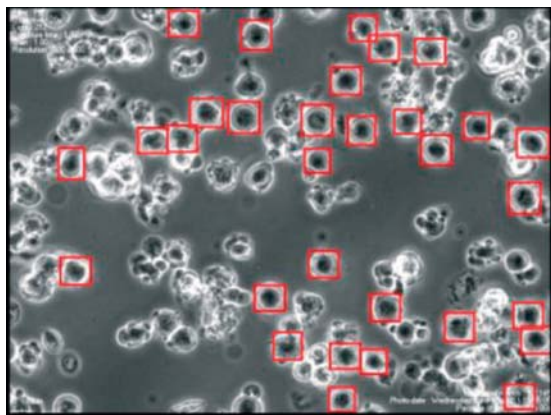


Figure 14. Detection of non-dynamic blebbing single unattached human embryonic stem cells (NDBSU-hESCs) in culture. Video bioinformatics tools are being developed to identify different classes of hESCs in culture. This image illustrates the accuracy of the program developed to detect this class of cells.

positive rate, while it gives a false positive rate $<5\%$. This method is currently being combined with additional methods for classifying all morphologically distinct cell types in mixed populations of stem cells.

Methods have been also been developed for stem cell colony detection and for detection of cells within a colony (Guan *et al.*, 2011, 2012a,b, 2013). Figure 15 provides an example of hESC colony analysis using an approach based on a mixture of Gaussians (Guan *et al.*, 2011). Figure 15a shows regions of interest, Figure 15b shows markers overlaid on the image, and Figure 15c shows the result after cell colony analysis. Good detection rates of individual cells within small colonies were achieved by this approach with low false alarm rates. Automated counting of cells matched favorably with the ground truth although the method overestimated the count slightly. This tool provides a way to count cell numbers in hESC colonies and can be applied to toxicological problems where changes in cell numbers are needed.

8.2 Future Directions

There is an urgent need to develop advanced techniques, fundamental algorithms, and technology, which will provide greater sensitivity, objectivity, and repeatability of experiments based on video data and to apply these methods to toxicological problems. This will make it possible for large volumes of video data to be efficiently analyzed and for fundamental questions in toxicological sciences to be answered. Biological characteristics are not fully exploited, computer tools are used only for very low-level analysis, and the process is highly human-intensive. One cannot simply use standard image processing, computer vision, or pattern recognition techniques and expect good results as the domain of biological videos has its own peculiar characteristics and complexities. Furthermore, the varying requirements of users dictate an integrated approach using machine learning rather than handcrafted user-specific solutions to individual problems.

9 CONCLUSIONS

Stem cells provide unique models for solving toxicological problems and will be extremely valuable

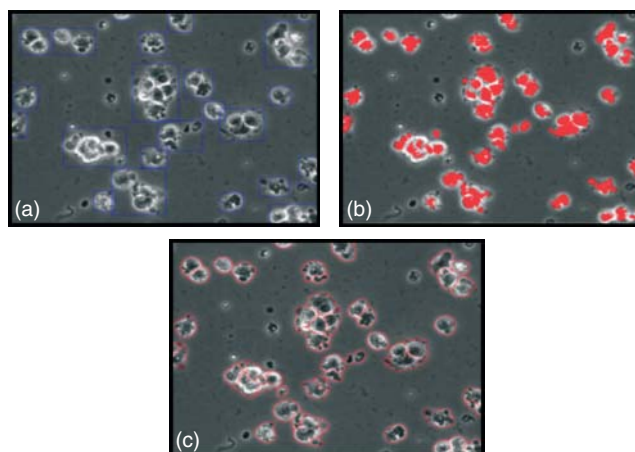


Figure 15. Detection of cells within hESC colonies. (a) Regions of interest. (b) Markers overlaid on the image. (c) The result after cell colony analysis. As shown in (c), the method accurately detects individual cells in small hESC colonies.

for understanding how chemicals and drugs affect human prenatal development as well as adult health. The introduction of VBI tools to stem cell applications in toxicology opens a new avenue for exploring the effects of chemicals on living cells, provides unique dynamic output acquired from living cells, and can be multiplexed to provide multiple end points in a single experiment. The use of stem cells in toxicological applications is a field in its infancy, and we expect to see dramatic advancements in this field in the future.

ACKNOWLEDGMENTS

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RELATED ARTICLES

Chapter 3
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Chapter 7

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Chapter 11

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 ICATM *see* International Co-operation on Alternative Test Methods
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 ICP-MS *see* inductively coupled plasma mass spectrometry
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